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AKADÉMIAI KIADÓ,
BUDAPEST 1969

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1969

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HISTOCHEMICAL STUDIES ON THE DISTRIBUTION OF ALKALINE AND ACID PHOSPHATASES IN THE BARBELS OF A FRESH-WATER TELEOST, SACCOBRANCHUS FOSSILIS

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(Received July 8, 1967)

Synopsis

In the present study the distribution of alkaline and acid phosphatases has been studied amongst the constituents of the barbels of *Saccobranchus fossilis*. The physiological significance of the distribution of these phosphatases in different cells types of the taste bud, viz., gustatory and supporting cells and a peculiar basal cell type described in this paper, is discussed in relation to the reception and the conduction of sensory impulses. The roles of the enzymes in other cellular constituents of the epithelium and central cartilaginous rod of the dermis are also discussed.

Introduction

No doubt the record of the histological studies on the barbels of various fishes is available in the literature [1, 4, 7, 11, 12, 13, 16, 22, 23, 24, 25], but neither the distribution of enzymes in the various constituents of the layers of barbels has been attempted, nor the histological study of the barbels of *Saccobranchus fossilis* has been made.

The present study attempts to record in some detail the distribution of alkaline and acid phosphatases in various layers of the barbels of a fresh water teleost, *Saccobranchus fossilis*.

Material and method

The living specimens of *Saccobranchus fossilis* were collected from local sources. Small pieces of the maxillary barbels were cut at their distal ends and were fixed in 10% neutral formalin or 85% alcohol or acetone for varying lengths of time. Fresh and fixed frozen sections as well as paraffin sections were cut at 10 μ. The sections were processed through Gomori's technique [17] for alkaline and acid phosphatases. Simultaneously suitable controls were made.

Results

General histology

The most remarkable external appendages existing on the head of the cat fish are four pairs of barbels, namely one pair each of nasal and maxillary and two pairs of mandibular barbels. Histologically all the four pairs of barbels are similar and are made up of two principal layers, viz., the epidermis and dermis. The epidermis consists of stratified epithelial cells followed by the basement

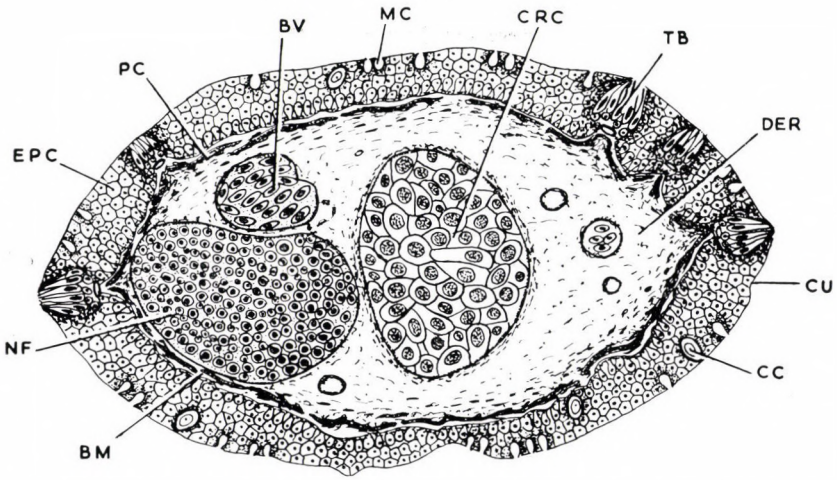


Fig. 1. Transverse section of the maxillary barbel of *Saccobranchus fossilis*. BM — basement membrane; BV — blood vessel; CC — club cell; CRC — central cartilaginous rod; CU — cuticle; DER — dermis; EPC — epidermal cells; MC — mucous cell; NF — nerve fibres; PC — pigment cell; TB — taste bud

membrane which separates it from the dermis. The epidermis contains a number of taste buds, mucous cells and club cells. The dermis is made up of connective tissue which is compact along the basement membrane and loose towards the inner side. Dermis contains a central cartilaginous skeletal rod with cartilaginous cells. The dermis is richly supplied with nerve fibres and blood vessels; a few pigment cells are also present along the basement membrane (Fig. 1).

Histochemical studies

Alkaline phosphatase: The epidermis of the barbel shows a positive reaction (EP; Fig. 2), in the dermis the central cartilaginous rod shows pronounced

activity in the nuclei (N; Fig. 8) of the cells and moderate activity in the cytoplasm (C; Fig. 8). The perichondrium layer surrounding the cartilaginous rod, however, provides an intense reaction (arrows; Fig. 8). In the epidermal cells the activity is present in the nuclear areas of the cells (N; Fig. 2). The cytoplasmic areas (C; Fig. 2) are negative. The thin cuticle covering the epidermis also shows the positive reaction (arrow; Fig. 2). Further, the enzymatic localization in the club cells (CC; Fig. 2) is similar to that in the cells of epidermal region, i.e., the reaction is restricted to the nuclear areas, the cytoplasm being totally negative.

In the taste bud two types of cells have been distinguished (gustatory or neuroepithelial cells and supporting or sustentacular cells, see Fig. 9). The nuclei of the gustatory cells show a positive reaction, while the cytoplasm of the cells is negative for the enzyme (GC; Fig. 4). According to BEIDLER [3] the apical processes of the gustatory cells are drawn out into hair-like processes and the tips of these processes bear microvilli. In our preparations the terminal portions of the fibres (encircled) have given an intense reaction as compared to the lower portions of the hair-like processes (arrows; Fig. 4). Like those of the gustatory cells the nuclei of the supporting cells are positive for alkaline phosphatase (SC; Fig. 4). One of the most important observations in the present studies is the identification of a peculiar type of cell (PBC; Fig. 4) at the extreme base of each taste bud. Such a type of cell has not been observed previously in the taste buds of other fishes studied by RAJBANSHI [21, 22, 23, 24] and other workers [4, 11, 25]. It is only mentioned in a report by CORDIER [8] dealing with the taste buds of *Coridoras hastatus* and *Macronus*. According to the present observation this cell possesses a long nucleus oriented transversely and a cytoplasm in the form of a thin rim. The nuclear area of this cell is completely devoid of the reaction in contrast to the positive nuclei of the gustatory and supporting cells (GC; SC; Fig. 4). On the other hand, in contrast to the cytoplasm of the gustatory and supporting cells, the cytoplasm of this basal cell is intensely positive for the enzyme.

Acid phosphatase: Similar to the disposition of the alkaline phosphatase the reaction of the acid phosphatase is also confined to the epidermal region of the barbel, while in the dermis the enzyme is restricted to the cartilaginous skeletal rod (Figs 3 and 6). Amongst the epidermal cells two patterns of distribution of the enzyme are to be distinguished. In some, the nuclei are positive and the cytoplasmic rims are distinctly negative (EPC_1; Fig. 3), in others the situation is just the reverse, i.e., the nuclear areas (N; EPC_2; Fig. 5) are negative and the cytoplasmic areas are positive (C; EPC_2; Fig. 5). The club cells (CC; Fig. 3) present in the epidermal region give positive reaction in the nuclei and the cytoplasmic areas are always devoid of the reaction.

Fig. 6 depicts a lower portion of a taste bud. In this section the peculiar basal cell (PBC; Fig. 7) exhibits a negative nuclear area and a positive cyto-

plasmic area. In the present observation a space (IGS; Fig. 7) above the peculiar basal cell has been observed. BARADI [2] observed such a space by light and electron microscopy in the taste buds of the dog and termed it intragemmal space. This space in the taste bud of *Saccobranchus* is totally devoid of acid phosphatase activity. The gustatory cells (GC; Fig. 7) provide a moderate reaction for acid phosphatase.

Several nerve fibres (both myelinated and unmyelinated according to FARBMAN [9]) are present at the base of the taste bud lower to the peculiar basal cell (NF; Fig. 7). The axons of these nerve fibres show positive reaction.

In the dermis the cartilaginous rod shows positive reaction (Fig. 6). In the cartilaginous rod the perichondrium layer is negative (arrows; Fig. 6) and in this respect it differs from the alkaline phosphatase preparations where such areas are intensely positive (compare arrows of Fig. 8 with those of Fig. 6). The nuclei (N; Fig. 6) of the cartilage cells are positive for acid phosphatase. On the other hand, the cytoplasmic areas of these cells are negative (C; Fig. 6).

Figs 2—8. Microphotographs representing 10 μ thick transverse sections of the maxillary barbel

Fig. 2. Alkaline phosphatase reaction in paraffin section. Note the intense positive reaction in the epidermis (EP), in contrast with the negative reaction in the dermis (DER). Note the reaction in the nuclei (N) of the epidermal cells; the cytoplasm (C) is negative. The nuclei of the club cells (CC) are also positive. Strong reaction is seen in the thin cuticle investing the epithelium (arrow). Positive nuclei (N_1) in the cartilage cells. C_1-cytoplasm of the cartilage cell. $\times 600$

Fig. 3. Acid phosphatase reaction in frozen section. Note the positive reaction in the nuclei of the epidermal cells (EPC_1) and those of club cells (CC). Dermis (DER) is devoid of the reaction. $\times 660$

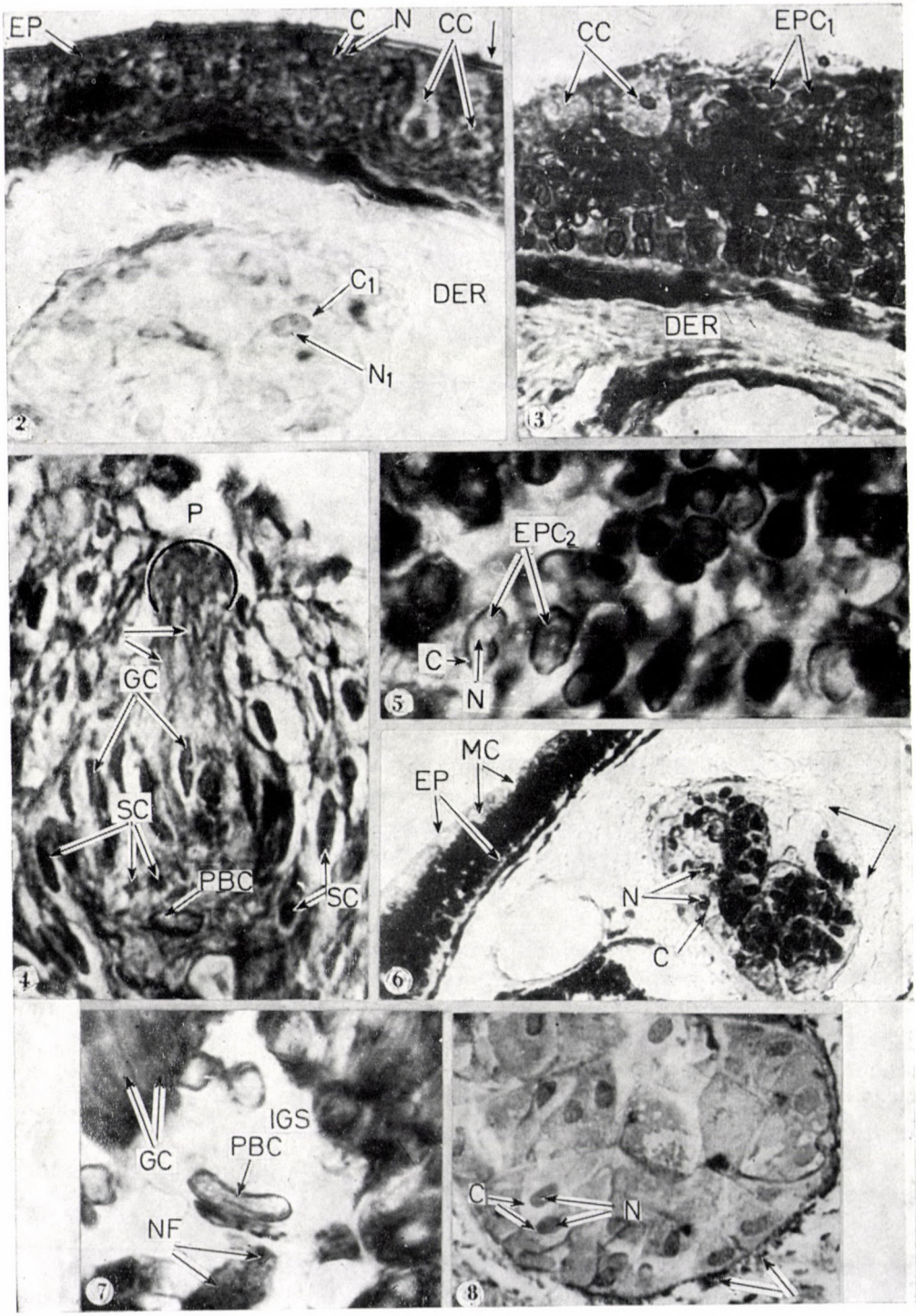
Fig. 4. Alkaline phosphatase reaction in taste bud (paraffin section). Distinguish the positive activity in the nuclei of the gustatory cells (GC) and supporting cells (SC). Positive reaction is seen to be extending in the middle portion of sensory hairs of the gustatory cells (arrows). Note the intense activity at the tips of sensory hairs (within circle) and the positive reaction in the cytoplasm of the peculiar basal cell (PBC). P = taste bud pore. $\times 1{,}120$

Fig. 5. A part of the frozen section showing acid phosphatase reaction in the epithelial cells (EPC_2). Note the positive reaction in the cytoplasmic rim (C) with negative nuclear area (N). Compare the reaction of these cells with the other epithelial cells where the positive areas are reverse (EPC_1; Fig. 3). $\times 1{,}330$

Fig. 6. Acid phosphatase reaction in frozen section. Note the intense positive reaction in the epidermis (EP). Mucous cells (MC) in the epidermal region are totally negative. The nuclei (N) of the cartilaginous cells show an intense positive reaction while the cytoplasmic areas (C) are negative. Distinguish the negative perichondrium (arrows). $\times 330$

Fig. 7. Acid phosphatase reaction in frozen section showing the lower half of the taste bud. Note the positive reaction in the cytoplasm of the peculiar basal cell (PBC). Gustatory cells (GC) are also positive. Note the positive reaction in the axons of the nerve fibres (NF) innervating the taste bud. Intragemmal spaces (IGS) seen in the taste bud represent a complete negative area. $\times 1{,}360$

Fig. 8. Alkaline phosphatase reaction in paraffin section showing central cartilaginous rod. Distinguish the positive activity of enzyme in the nuclei (N) of the cartilage cells while the cytoplasmic area (C) shows moderate reaction. Note the concentration of enzyme at the perichondrium layers (arrows) of the cartilage. $\times 630$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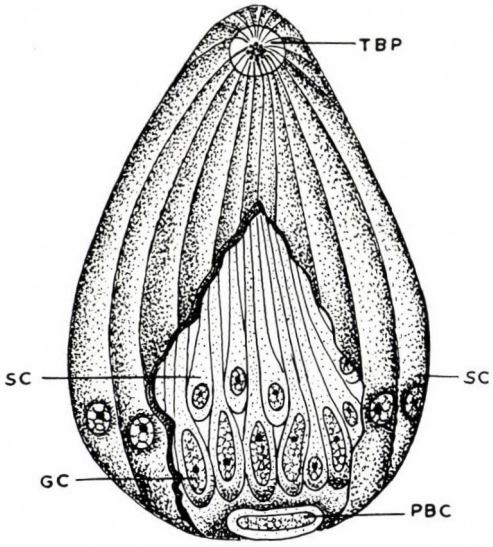

Fig. 9. Diagram of the taste bud showing the arrangement of the cells inside the taste bud. GC — gustatory cells; PBC — peculiar basal cell; SC — supporting or sustentacular cells; TBP — taste bud pore

Discussion

In all the epidermal cells (including the club cells) the alkaline phosphatase activity is located in the nuclei. In these areas the enzyme may be concerned in nucleic acid synthesis. A number of workers [28, 29] have pointed to such a possibility in other cells. Regarding the acid phosphatase localization in the epidermal cells, the pin-point significance seems difficult to approximate as some cells have shown positive reaction in the nuclei and other cells in the cytoplasm. Presumably the manifestation of the acid phosphatase activity in respective zones may indicate an activity of the enzyme in nucleic acid synthesis [28, 29] occurring in the nuclei and cytoplasm, respectively, at different times. Such a role can also be associated to alkaline phosphatase in the areas such as the nuclear sites of the supporting and gustatory cells. However, the significance of the absence of the alkaline and acid phosphatases in the nuclei of the peculiar basal cells is difficult to interpret.

As to the significance of the alkaline phosphatase in the sensory hair-like processes of the gustatory cells, VINNIKOV and TITOVA [30] suggest that the localization of phosphatases in the sensory cells indicates their role in a number of physiological functions, particularly in the excitation and transmission of nerve impulses. BOURNE [6] and TEWARI and BOURNE [26, 27] in various

regions of the brain have also associated the role of phosphatases with the processes playing part in the transmission of nerve impulses. In the present case the localization of the alkaline phosphatase in a continuous pattern from the region of the tips of the hair processes (constituting the microvilli studied by electron microscopy by FARBMAN [9]) to the gustatory cells through the entire region of the hair-like processes seems to indicate the participation of the enzyme in the conduction of the nerve impulse.

In the sensory cells of the lateral line organs FLOCK and WERSÄLL [10] have also described a sensory hair composed of a large kinocilium and about fifty small sterocilia. In the taste buds of the members of all classes of vertebrates PEVZNER [18, 19, 20] have described the kinocilia of the gustatory cells which have become modified into "shafts". These shafts, according to her, are equipped with total protein, mucopolysaccharides and alkaline phosphatase. She further points out that the shaft plays the part of an ion-exchanging appliance interposed between gustatory substances and the receptor cells. In the present observation it is quite likely that the alkaline phosphatase in the sensory hairs may also play a similar role.

It has been suggested that the alkaline phosphatase reaction at the perichondrium layer (fibrous layer) of the central cartilaginous rod plays some role in calcification [14, 15]. Calcification starts at the periphery of the cartilage cells. The granules of calcium which are first formed at the peripheral sites later coalesce and form a series of crystalline plates [5]. In the present case the peripheral localization of the alkaline phosphatase seems to signify its participation in the process of calcification occurring at the peripheral sites. The localization of alkaline and acid phosphatases in the nuclei of the cartilage cells may be concerned with the nucleic acid synthesis similar to the processes occurring in other cells of the epithelium.

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HISTOCHEMISCHE UNTERSUCHUNG DER VERTEILUNG DER ALKALISCHEN UND SAUREN PHOSPHATASEN IN DEN FÜHLERN VON FISCHEN *SACCOBRANCHUS FOSSILIS*

Verfasser ermittelten die Verteilung der alkalischen und sauren Phosphatasen in den Fühlern von *Saccobranchus fossilis.* Die physiologische Bedeutung ihrer Verteilung in den verschiedenen im vorliegenden Artikel beschriebenen Zellentypen der Geschmacksknospe, u. zw. in den Geschmacks- und Stützzellen, sowie in einem besonderen Basalzellentyp wird in Zusammenhang mit der Wahrnehmung und Weiterleitung von sensorischen Impulsen besprochen. Die Rolle der Enzyme in anderen Zellbestandteilen des Epithels und im zentralen knorpeligen Stäbchen der Haut werden gleichfalls erörtert.

ГИСТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ РАСПРЕДЕЛЕНИЯ ЩЕЛОЧНОЙ И КИСЛОЙ ФОСФАТАЗ В УСИКАХ СВЕЖИХ *SACCOBRANCHUS FOSSILIS*

Проведено определение распределения щелочной и кислой посфатаз среди составных частей усиков ***Saccobranchus fossilis***. Физиологическое значение распределения этих фосфатаз в различных типах клеток вкусовой почки, а именно в вкусовых и опорных клетках и особый тип базальных клеток, описанных в настоящей статье, обсуждаются в связи с восприятием и проведением чувствительных раздражений. Обсуждается также роль энзимов в других клеточных элементах эпителия и центральных хрящевых палочках кожи.

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A COMPARATIVE STUDY OF THE EFFECTS OF CERTAIN CHEMICAL AGENTS ON CHROMOSOMES

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Synopsis

Root tips of eight varieties of common pulses belonging to the genera *Cicer*, *Pisum*, *Lens* and *Lathyrus* were subjected to treatment with colchicine, hexa-chloro-cyclohexane and chloral hydrate solutions at various concentrations and for various periods. The immediate effect of these chemicals and the behaviour of the root tips reviving in Knop's solution were studied. Colchicine was found to be effective for all these cases, hexa-chloro-cyclohexane is also effective for at least some cases, but chloral hydrate is mostly ineffective as it exerts some injurious effects on the tissue. With regard to the concentration, the effect of colchicine is more or less similar in prolonged treatments with different concentrations, whereas the same has been observed in other cases. Long periods of treatment in all cases lead to injurious effects but the degree of injury caused by chloral hydrate treatment is the highest, followed by that of hexa-chloro-cyclohexane. Though colchicine and hexa-chloro-cyclohexane are antimitotic agents, they allow at least a certain percentage of cells arrested in the metaphase stage to enter the metabolic stage and to divide as shown by the presence of certain polyploid cells in prolonged direct treatment with these chemicals. Such behaviour cannot be observed with chloral hydrate. Temperature in all the cases of recovery experiments exerts similar effects, viz., high temperature, in general, accelerates the effects of the chemicals. In all the cases specific differences were observed in relation to the susceptibility to these chemicals.

Introduction

The importance of polyploidizing effect of colchicine has been established by different workers [1]. Later, a number of other compounds have been demonstrated to possess the capacity of inducing polyploidy. Two of these, viz., chloral hydrate [5, 6] and hexa-chloro cyclohexane or Gammexane [2, 3, 4, 7], have been tried widely.

As the efficacy of different compounds often varies with different conditions, a study of the polyploidizing capacity of these compounds in a comparative basis on different plant species appears to be necessary. Since their use principally lies in agriculture and horticulture, it was deemed desirable to carry out this comparative experiment on plants of economic importance.

In the present investigation, therefore, the comparative effects of colchicine, chloral hydrate and Gammexane have been tried on each of the eight different species and varieties of commonly used pulses belonging to the genera *Cicer*, *Pisum*, *Lathyrus* and *Lens*.

Material and method

Some varieties of common pulses were selected as experimental materials. They are

1. *Cicer arietinum* var. Bengal, *Cicer* var. 1
2. *C. arietinum* var. Patnai, *Cicer* var. 2
3. *C. arietinum* var. Arab, *Cicer* var. 3
4. *Pisum sativum* var. Kabul, *Pisum* var. 1
5. *P. sativum* var. Kashmir, *Pisum* var. 2
6. *Lathyrus sativus*
7. *Lens esculenta* var. macrosperma, *Lens* var. 1
8. *L. esculenta* var. microsperma, *Lens* var. 2

The seeds were soaked in water and then were sown in plots containing sand and sawdust. When the radicles were 2 to 3 cm in length, the germinating seeds were taken for treatment. The following three sets were made for experiments.

Set I. (A) Intact roots were treated with aqueous colchicine solution of different concentrations — 0.5%, 0.25% and 0.1% — and for different periods — 4 hours, 6 hours and 24 hours. (B) Intact roots were treated with 0.25% colchicine solution for 4 and 24 hours and then allowed to revive in Knop's solution at room temperature (on average 28 °C) and at 14 °C.

Set II. (A) Roots were treated with aqueous chloral hydrate solution. The concentrations and periods of treatment were similar to set IA. (B) Roots were initially treated with 0.25% chloral hydrate for 4 and 24 hours and then allowed to revive in Knop's solution at 28 °C and 14 °C.

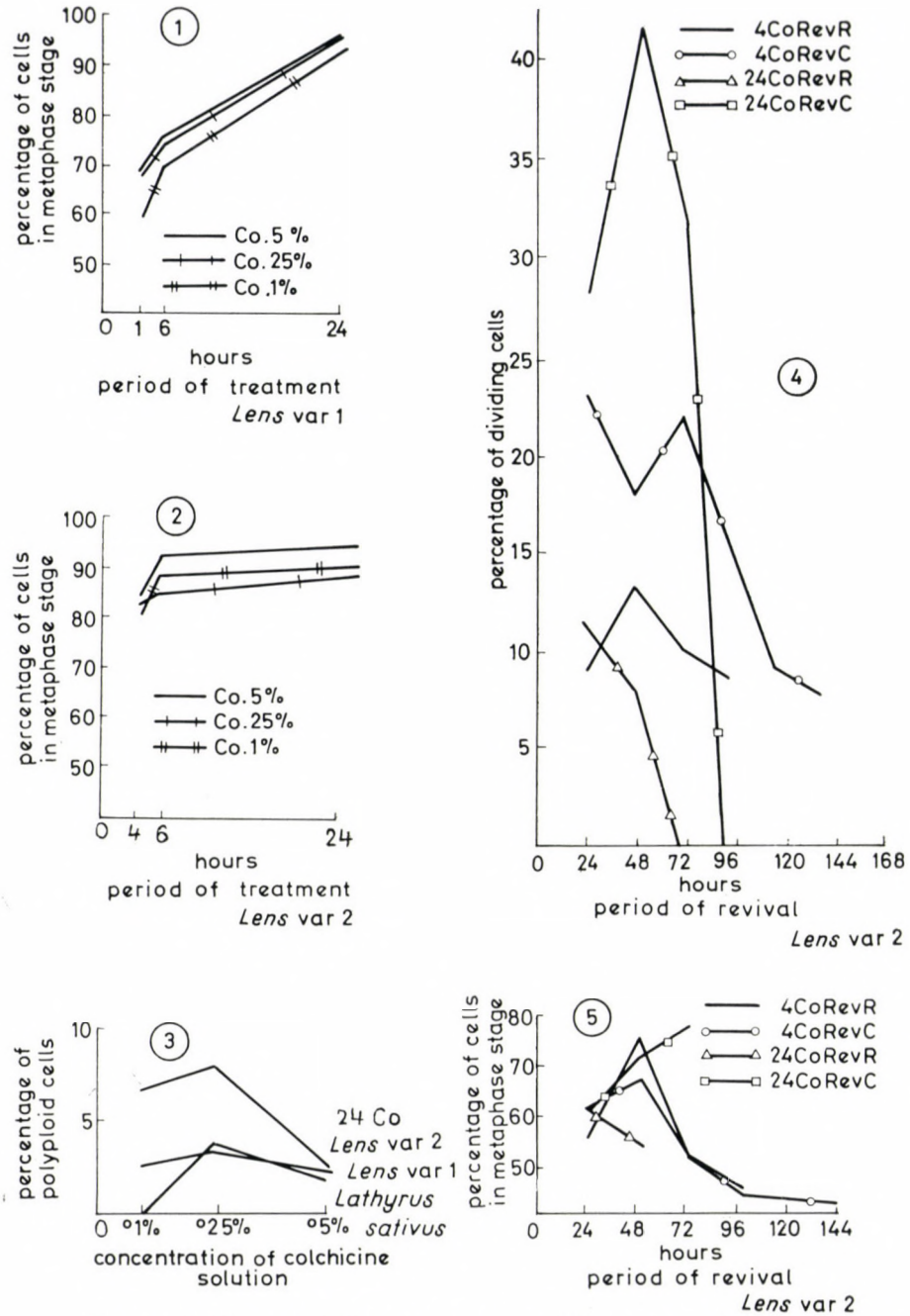
Set III. (A) Root tips were treated with half-saturated and saturated hexa-chlorocyclohexane solutions in water. The periods of treatment were 5 and 24 hours. (B) Experiments were made on revival of the root tips in Knop's solution after being treated with Gammexane solution of half-saturation and saturation strengths and for periods of 5 and 24 hours at temperatures of 28 °C and 14 °C.

The root tips were dipped in tubes containing the solutions for treatment in such a manner that cotyledons remained above the solution level. After the scheduled periods of treatment, they were taken out and washed in running water, after treatments for 4, 5 or 6 hours for about one hour, after 24 hours treatments for about 4 hours. To observe the immediate effect, some roots were cut out and fixed in acetic-alcohol (1 : 2). The other roots still attached to the seedlings were allowed to revive in Knop's solution at different temperatures. Roots were cut out after every 24 hours and were fixed in acetic-alcohol (1 : 2) mixture prior to observation.

Temporary preparations were made by squashing the root tips in 1% aceto-orcein solution after heating in 2% aceto-orcein and *N* HCl (9 : 1) mixture and keeping there for one hour. The squashes were made permanent by inverting the slides in butyl alcohol grades and finally mounting in euparal.

Results

Colchicine treatment. Roots are normal in earlier stages but show tumours after prolonged treatment, leading to injuries to some roots (Figs 1—6). The division frequency of metaphase arrest shows an increase in all cases, both with prolonged treatments and treatment for short period but with high concentration. In *Cicer* and *Pisum* varieties and in *Lathyrus* sp. the frequency of dividing cells is generally low but shows significant rise with the period of treatment. The *Lens* varieties, however, show a very high divisional frequency amongst which the rise is the highest in *Lens* var. 2. In *Pisum* and *Cicer* varieties and *Lathyrus* sp. frequency of metaphase is moderately high, whereas in *Lens* varieties a very high frequency of metaphase arrest is noted, particularly in those treated with high doses. In almost all the cases roughly identical results are



Figs 1—5. Effect of colchicine treatment on different species of pulses

observed with prolonged treatment for 24 hours, irrespective of the concentrations used, 0.5% or 0.1%. However the differences observed in the above cases are related to other abnormalities rather than to normal colchicine action. Treatments with high concentrations for a long time show injurious effects on the root tips and on the cells, leading to granulation of chromosomes, pycnosis and to flaccidity of roots in some cases.

In *Cicer* vars 1 and 3, *Lathyrus* sp. and in both varieties of *Lens* polyploid cells are obtained after 24 hours treatment, although the frequency is very low. The effects of different concentrations with different periods of treatment on different materials have been compared.

The results obtained from experiments on revival in Knop's solution both at 28 °C and 14 °C show the appearance of tumours in all the root tips in general. Tumours appear in lower percentage in the root tips treated for 4 hours. In many cases the reviving root tips become flaccid after 24 hours treatment and many roots show no sign of revival. Tumours are more developed in the roots reviving at 28 °C, but many of these roots do not recover at all. In these roots, secondary roots come out earlier than in the roots reviving at 14 °C.

The divisional frequency, in general, is much higher in roots reviving for 24 hours and then decreases to the normal frequency. In *Cicer* var. 2 root tips treated for 4 hours and revived at room temperature show a very low divisional frequency. At 72 hours no division can be found and after that period a high frequency of dividing cells can be observed. In the roots reviving at 14 °C, however, the frequency is very high from the beginning. In the roots treated for 24 hours and reviving at 28 °C and 14 °C, no division could be found after 48 and 72 hours, respectively. In *Pisum* var. 2 the same action is observed. In *Lens* var. 2 no division was observed after 72 hours in both the cases in which the roots were initially treated for 24 hours.

The frequency of cells in metaphase stage is found to be very high in the first stages of revival, particularly in the roots treated for 24 hours. In all the cases the frequency decreases with recovery period. A similar result can be observed with the frequency of polyploid cells, associated with the decrease in recovery period. Associated with the decrease in ploidy, various cytological processes, such as non-disjunction and multipolarity, are observed. In most cases of revival in cold the higher frequency of polyploid cells persists for long periods, although, in the roots reviving at 28 °C a higher percentage of polyploid cells is observed in the initial stage. In the latter case, the polyploid cells are eliminated either by various cytological processes or by the formation of secondary roots, where, in general, polyploid cells are absent. In one variety each of *Pisum*, *Cicer*, *Lens* and *Lathyrus* sp. a high frequency of polyploid cells persists even after 7 days of revival.

In the reviving roots other cytological abnormalities noted are contraction of chromosomes, diplochromatids, bright staining of the heterochromatin

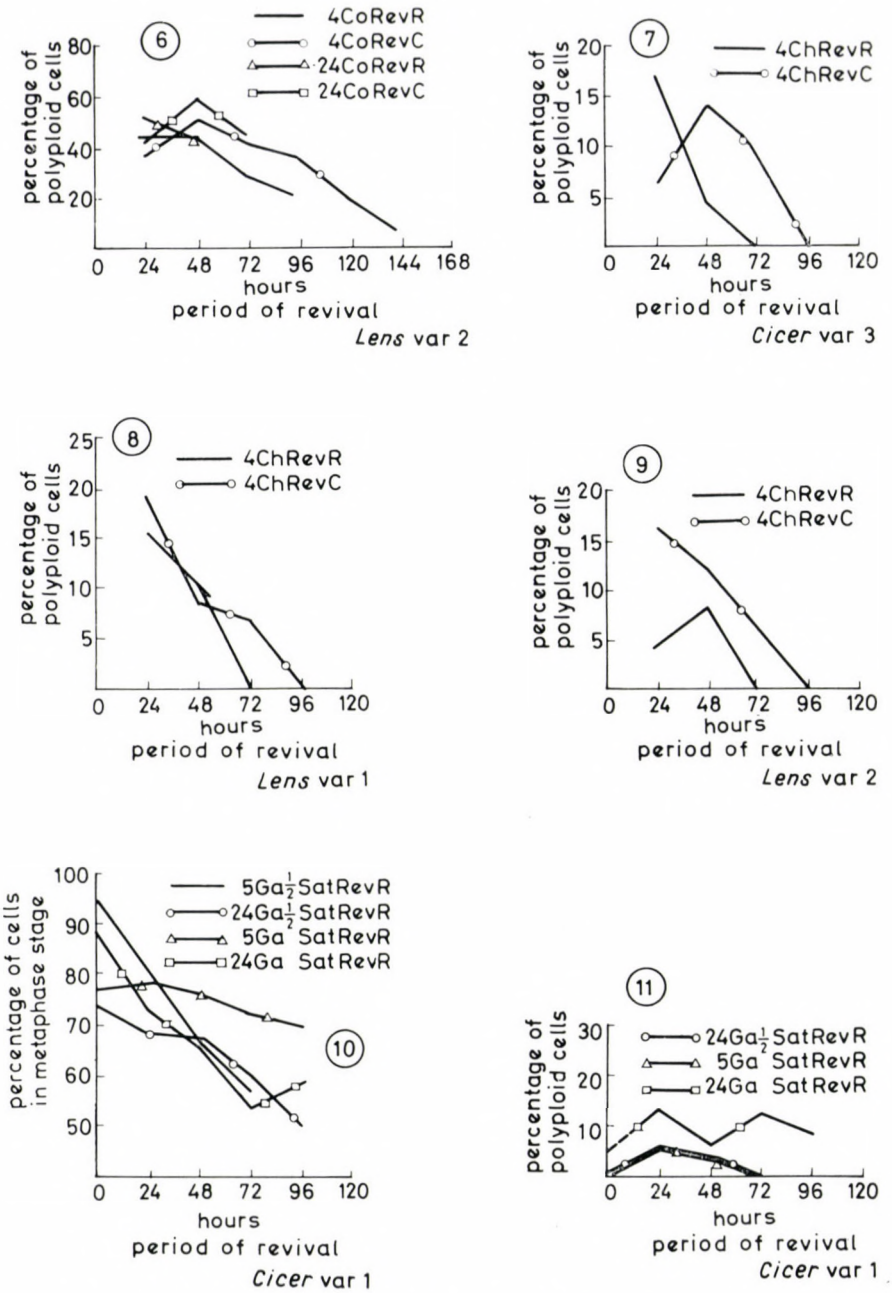


Fig. 6. Effect of colchicine treatment on different species of pulses
Figs 7—9. Effect of chloral hydrate on different species of pulses
Figs 10—11. Effect of hexa-chloro-cyclohexane on different species of pulses

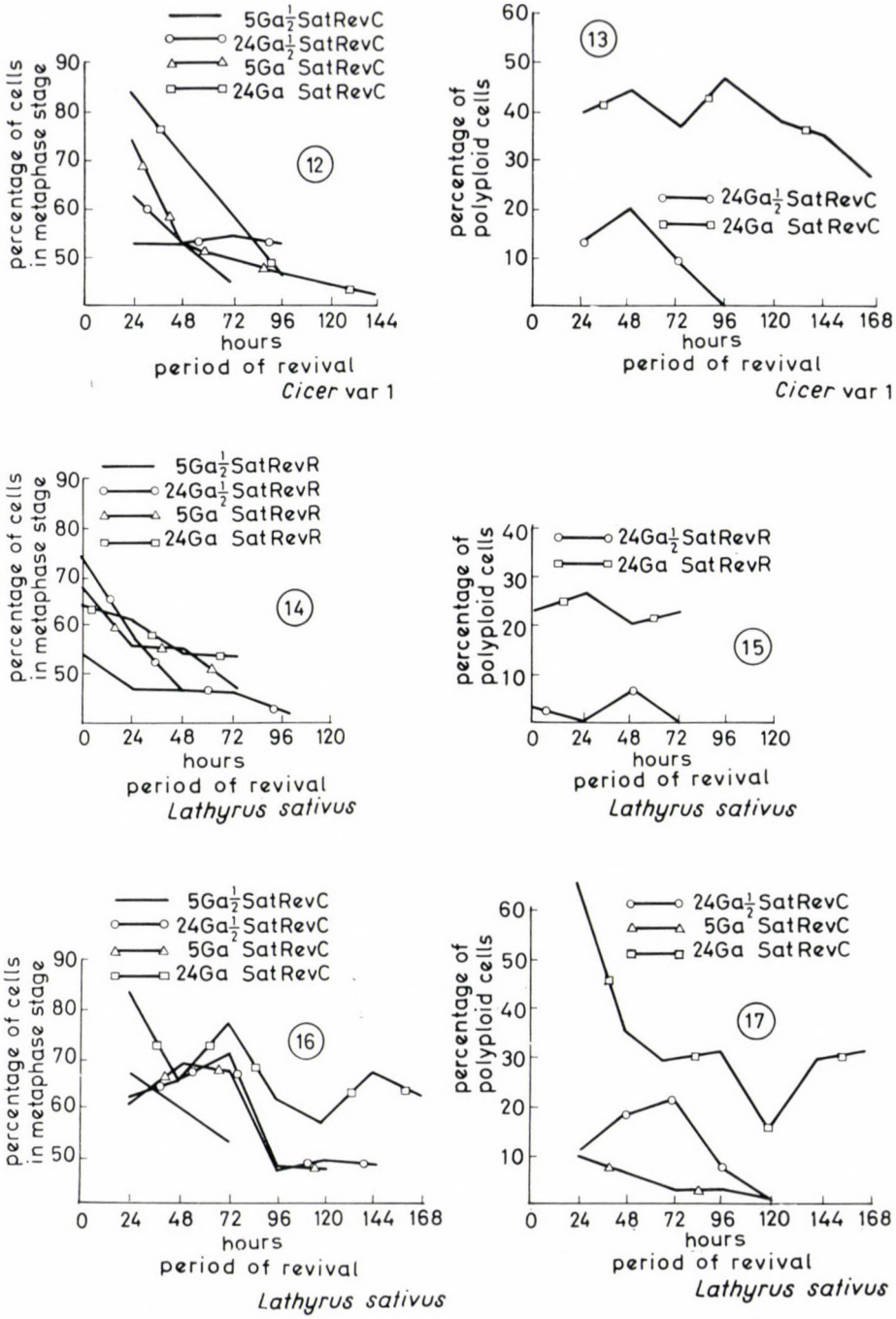
in the resting nuclei, clumping, stickiness, sticky bridge, lagging and fragmentation.

The frequency of dividing cells, cells in metaphase stage and polyploid cells in the reviving roots of different materials has been compared.

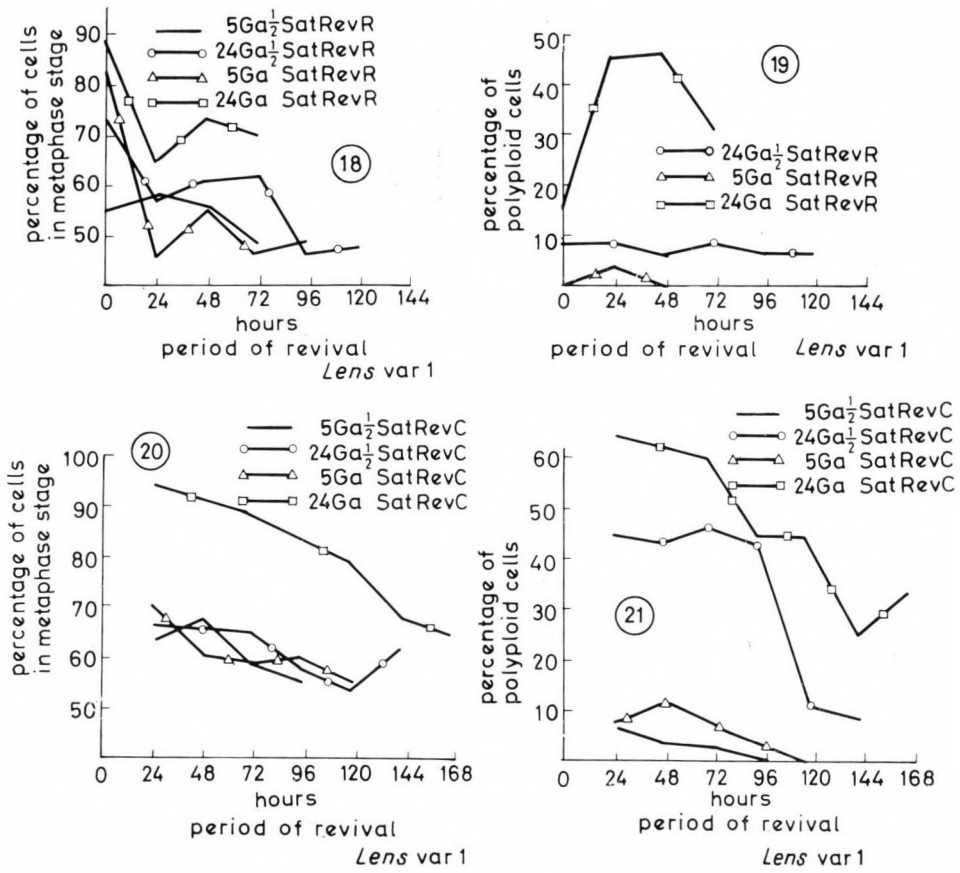
The results obtained in the roots of different materials treated with different concentrations of chloral hydrate solution show a slight increase in the divisional frequency in all the materials (Figs 7—9) but in *Cicer* var. 1 and *Lens* var. 1 the rise in the frequency of dividing cells, after 24 hours treatment, is much higher than in the others. In other materials 24 hours treatment in 0.5% or 0.25% chloral hydrate solution proves to be lethal. In *Lens* var. 2 and *Pisum* var. 2 the frequency of division is significantly low with some treatments. In *Lens* vars 1 and 2, and *Pisum* vars 1 and 2 the frequency of cells in metaphase stage is very high at some dosages. Definite polyploid cells could not be observed except in an isolated case in *Pisum* var 1. Other abnormalities associated are clumping, erosion, stickiness, sticky bridge and fragmentation. Heterochromatin is brightly stained in *Cicer* vars 1 and 2 on prolonged treatment. Granulation of chromosomes can be observed with prolonged treatment in those cases where the dosage does not prove to be lethal. In general, however, contraction of chromosomes associated with appearance of diplochromatids is very common.

Results on the revival experiments show in most cases except in *Cicer* var. 1 and *Pisum* var. 1 that roots treated for 24 hours do not revive at all. In *Cicer* var. 1 and *Pisum* var. 1, however, the roots treated for 24 hours do not revive at 28 °C, whereas a low percentage of the roots reviving in cold show signs of recovery. Tumours develop in roots of *Lens* var. 1 and *Cicer* var. 3 initially treated for 4 hours and in *Pisum* var. 1 initially treated for 24 hours. In the last two cases only significantly high frequency of dividing cells can be observed. In other cases, although a slight rise in the frequency of dividing cells is observed, yet it falls to normal within a short period. The frequency of cells in metaphase stage is nearly parallel with that of dividing cells. A significantly high frequency of polyploid cells is observed in *Lens* vars 1 and 2 and *Cicer* valr. 3. Occasional polyploid cells can be observed in *Cicer* var. 1, *Pisum* var. 1 and in *Lathyrus* sp. In none of the cases can polyploid cells be observed after 96 hours revival. Other abnormalities observed in the reviving roots are fragmentation, stickiness, clumping, lagging, early separation and contraction of the chromosomes. In the roots where polyploid cells are found, multinucleate cells, multipolarity, non-disjunction of chromosomes, etc., are associated with the reduction of chromosome numbers.

The results of hexa-chloro-cyclohexane treatment on the roots of different materials and their subsequent revival in Knop's solution at 28 °C and results of revival experiments at 14 °C are given in Figs 10—21. In all these cases, a higher frequency of dividing cells can be observed in roots reviving at 14 °C than in those reviving at 28 °C. Within two varieties of *Lens*, *Cicer* var. 1 and



Figs 12—17. Effect of hexa-chloro-cyclohexane on different species of pulses



Figs 18—21. Effect of hexa-chloro-cyclohexane on different species of pulses

Pisum var. 1, the divisional frequency is remarkably high in reviving roots initially treated with saturated solution. In these cases, however, the frequency is not as high as in the colchicine-treated roots. Half-saturated hexa-chloro-cyclohexane solution can produce little effect within 5 hours, although in a few instances treatment for the same period with saturated solution produces significant results. The roots treated with saturated solution for 24 hours produce much higher effect than that produced in the roots treated with the half-saturated solution for the same period.

The frequency of cells in metaphase stage is high immediately after the treatment with high dosages but falls rapidly during the revival period. But the frequency is never as high as that obtained with colchicine.

After treatment with half-saturated solution for 5 hours and then revival at 28 °C, polyploid cells can be observed only in two varieties of *Lens*. A significantly high frequency of polyploid cells is observed in all the roots treated with

saturated solution for 24 hours and then reviving in Knop's solution. In *Cicer* var. 1 and 2 and in *Lathyrus* sp. a high frequency of polyploid cells can be observed even after a long period of recovery at 14 °C, whereas roots reviving at 28 °C show much lesser effects. In these cases also the initiation of secondary roots is earlier in the roots reviving at 28 °C than in those reviving at 14 °C. The roots reviving at the higher temperature often show some other injuries.

Associated with these effects other abnormalities noted are fragmentation, micronuclei formation, non-disjunction, lagging, early separation, etc. Multipolarity and multinucleate conditions are also observed associated with the reduction of chromosome numbers of the polyploid cells.

Discussion

Results so far obtained from treatments with colchicine, chloral hydrate and hexa-chloro-cyclohexane have revealed certain interesting differences between the effects of these drugs.

Colchicine has been found to be the most effective in almost all the species treated. However, differences in susceptibility have been brought out between species and in this respect species of *Lens* show much more susceptibility as compared to the rest of the pulses (Figs 1, 2 and 3). Of the rest, *Lathyrus sativus* shows higher sensitivity.

In relation to colchicine effect, though in periods of treatment, concentration of the solution was found to be an important factor controlling the degree of the effect, yet with longer periods of treatment nearly similar results were obtained in different concentrations.

Though an antimitotic agent, colchicine, in addition to causing metaphase arrest, allows at least a certain percentage of cells to divide further, as shown by the appearance of some polyploid cells in direct colchicine treatment without undergoing any recovery. This is only possible if the cells after arrest in metaphase, enter further into metabolic stage and enter in the subsequent divisions.

Moreover so far as the persistence of the effect is concerned, temperature exerts a significant influence on the process. At 28 °C the effects do not persist further as the tissue ultimately shows pycnosis and dies. Cold temperature, on the other hand, accelerates the persistence significantly. A remarkable instance of differential sensitivity was observed in case of *Lens esculenta* var. microsperma which unlike its related variety shows signs of activity very early in development of the root and ultimately dies, even when kept at a low temperature (Figs 4—6).

The above aspects of colchicine action show certain differences from those of chloral hydrate and hexa-chloro-cyclohexane. So far as chloral hydrate is

concerned, though metaphase arrest could be secured in almost all the species, subsequent polyploid after recovery could not be observed except in only a few cases as *Lens* vars 1 and 2 and *Cicer* var. 3 (Figs 7—9). Moreover, unlike colchicine, it does not allow any further division after metaphase arrest indicated in the absence of any polyploid cells in prolonged direct treatment. Further, the toxicity exerted by chloral hydrate is rather remarkable in the sense that lethality ensues even in recovery medium within a short period. Heavy susceptibility of *Lens esculenta* and *Cicer arietinum* var. 3 as compared to the rest of the species in chloral hydrate treatment is noted in the appearance of polyploid tumours. In general the low persistence of the effect and toxicity of the effect show that chloral hydrate is a much less efficient agent in inducing polyploidy in pulses. So far as the temperature effect is concerned the behaviour is, however, the same as that of colchicine.

At last, efficiency of hexa-chloro-cyclohexane must be considered as more than that of chloral hydrate. Like colchicine, hexa-chloro-cyclohexane though an antimitotic agent allows a low frequency of nuclei to divide further even when the tissue is kept in the solution. This behaviour, though common with colchicine, differentiates it from that of chloral hydrate. Further, unlike chloral hydrate, it does not exert a strong toxic effect on the tissue and the effects persist for a long period in the recovery medium. In this respect here also cold temperature was found to accelerate the effects. Susceptibility to hexa-chloro-cyclohexane is specially high in *Cicer* var. 1, in *Lathyrus* and *Lens* var. 1 (Figs 10—21), where a high frequency of polyploid cells was maintained in the root cells after a long period of recovery. This is another instance of the difference in sensitivity of different species to chemical treatment.

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VERGLEICHENDE UNTERSUCHUNG DER WIRKUNG VERSCHIEDENER CHEMIKALIEN AUF DIE CHROMOSOMEN

Die Wurzelspitzen von 8 Hülsenfrüchtenarten der Gattungen *Cicer*, *Pisum*, *Lens* und *Lathyrus* wurden mit Colchicin, Hexa-chloro-cyclohexan- und Chloralhydrat-Lösungen verschiedener Konzentrationen verschieden lang behandelt. Verfasser untersuchten den unmittelbaren Effekt dieser Behandlung und die Wiederbelebung der Wurzelspitzen in Knopscher Lösung. Colchicin erwies sich in sämtlichen Versuchen als wirksam. Hexa-chloro-cyclohexan war in einigen Fällen effektiv, während Chloralhydrat zumeist unwirksam blieb, da es eine schädigende Wirkung auf das Gewebe ausübt. Die Colchicinwirkung war bei langdauernder Behandlung mit verschiedenen Konzentrationen mehr oder weniger gleich, während mit den anderen Chemikalien dies nicht beobachtet wurde. Langwährende Behandlung führte in sämtlichen Experimenten zu Schädigungen, das höchste Ausmaß erreichten sie bei Chloralhydratbehandlung, und die nächstschwersten Schädigungen erzeugte Hexa-chloro-cyclohexan. Obwohl Colchicin und Hexa-chloro-cyclohexan antimitotische Wirkstoffe sind, lassen sie es dennoch zu, daß zumindest ein gewisser Prozent der im Stadium der Metaphase arretierten Zellen in die metabolische Phase tritt und sich teilt; das Vorhandensein einiger polyploider Zellen bei prolongierter Behandlung mit diesen Chemikalien stützt diese Annahme. Die gleiche Erscheinung kann bei der Verwendung von Chloralhydrat nicht beobachtet werden. Die Temperatur rief in sämtlichen Wiederbelebungsversuchen die gleichen Wirkungen hervor. Hohe Temperaturen erhöhten in der Regel die Wirkung der Chemikalien. In allen Versuchen wurden spezifische Unterschiede in der Empfindlichkeit gegenüber den untersuchten Chemikalien beobachtet.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ДЕЙСТВИЯ НЕКОТОРЫХ ХИМИЧЕСКИХ ВЕЩЕСТВ НА ХРОМОСОМЫ

Верхушки корней восьми видов бобовых из родов *Cicer*, *Pisum*, *Lens* и *Lathyrus* подвергались обработке растворами колхицина, гексахлорциклогексана и хлоралгидрата при различных концентрациях и с различной продолжительностью. Исследовалось непосредственное действие этих химических веществ на восстановление верхушек корней в растворе Кнопа. Колхицин оказался эффективным во всех случаях: гексахлорогексан также был эффективным, по крайней мере в нескольких экспериментах, но хлоралгидрат в большинстве случаев не давал эффекта, так как оказывал некоторое вредное действие на ткани. При длительной обработке с различными концентрациями колхицин оказывал более или менее одинаковое действие, в то время как в случае применения других веществ этого не наблюдалось. Длительная обработка во всех случаях приводила к повреждению тканей, причем самое сильное повреждающее действие оказывал хлоралгидрат, а второе место в этом отношении занимает гексахлорциклогексан. Хотя колхицин и гексахлорциклогексан являются антимитотическими химическими веществами, они в некотором проценте клеток допускают переход из стадии метафазы в метаболическую стадию и деление; это доказывается наличием нескольких полиплоидных клеток при длительной непосредственной обработке этими средствами. В случае применения хлоралгидрата этого не наблюдается. Температура во всех экспериментах по восстановлению корней оказывала одинаковые эффекты. Высокая температура, в общем, повышает действие исследовавшихся химических веществ. Во всех экспериментах наблюдались специфические разницы в чувствительности к этим химическим веществам.

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REPORT OF AN ALGAL BLOOM IN VIET-NAM

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Synopsis

On 25th January, 1966, on a wet winter day with drizzle of the winter monsoon, during frontal passage, an algal bloom of vivid green colour was developed in the Little Lake of Hanoi at a water-temperature of +13 to 26 °C. The Little Lake, an isolated backwater of the Red River traversing Hanoi, contains contaminated water. The algal bloom was unusually rich in species and new taxons. The number of the taxons participating in the algal bloom was 81. Qualitatively, the Cyanophyta and the Chlorophyta were dominating. Variability of the *Scenedesmus* species was especially prominent. In a bottom sample drawn from the same place 115 taxons — without the Bacillariophyceae — have been identified. Also here, most of the taxons belonged to the Cyanophyta and the Chlorophyta. The number of the *Scenedesmus* species overpassed that in the algal bloom. Quantitatively, both in the algal bloom and the benthos the Cyanophyta were dominating with 82% in the algal bloom and 92% in the bottom sample, followed by Chlorophyta with 12% and 6.8%, respectively. Between the two biotops no definite line of demarcation can be found because the algal bloom had developed above the bottom and the bottom plants are easily raised near to the surface by the waves, convectional streamings and the movements of fishes. Nevertheless the algae of the two biotops are clearly different, the phytobiocoenoses being not the same. The number of common taxons is 66, i.e. 51% of all the taxons. The number of undescribed taxons was 33 including 30 *Scenedesmus* species. The presence of several organisms deserves attention. The parallel morphoses are especially well represented by some *Scenedesmus* species. It is remarkable that, in Hanoi, among the spiny forms of the Chlorococcales those of thin spines are less frequent. It seems that towards the tropics spines, ribs, globules and protuberances become more and more developed.

Introduction

In January of 1966, after a rain-front, on a stuffy, wet day with drizzle of the winter monsoon, at a water-temperature of +13—26 °C, an algal bloom of vivid green colour was developed in Hanoi, in the Little Lake. This lake is a backwater of the Red River isolated long time ago. The lake is situated at the 21st degree of the north latitude. The city of Hanoi has totally surrounded it. The shore of the lake is gently sloping, loamy, planted with trees and paved only here and there. Its inundation soil is loamy. A great quantity of sewage flows into the lake from the surrounding houses.

The material examined by us was a water-sample of the algal bloom taken in January obtained from the alga-covered mud of the lake-bottom at a distance of a metre from the shore. I express my thanks to Mr. T. Pócs for the samples.

Results and discussion

Qualitative conditions

In that algal bloom unusually rich in species and new taxons 81 took part, viz.: Cyanophyta 18 (22.2%); Euglenophyta 1 (1.3%); Chlorophyta 60 (74%); Caulobacteriales 2 (2.5%). The phylum Chlorophyta was represented by Chlorococcales (58 = 71.5%), and Conjugatophycaea (2 = 2.5%). Therefore blue-green algae and green algae were qualitatively dominating. Among Cyanophyta the species *Gloeocapsa granosa* and *Merismopedia* and *Microcystis* species were frequent. Among the green algae *Ankistrodesmus*, *Pediastrum*,

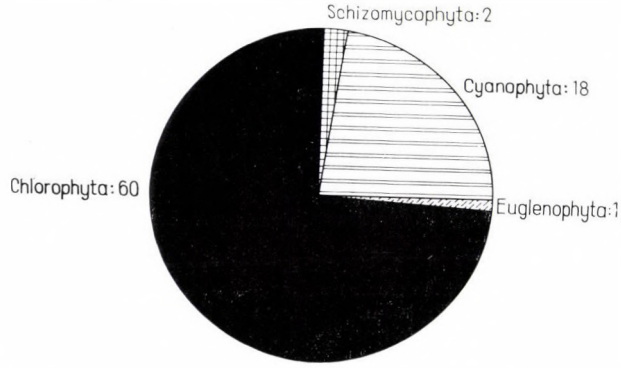

Fig. 1. Qualitative diagram of the plants in the algal bloom of Hanoi. The numbers beside the names of the phyla indicate the taxon-number

Scenedesmus and *Tetraëdron* species are to be mentioned; especially, the *Scenedesmus* species show a conspicuous variability. In the algal bloom 31 *Scenedesmus* taxons, i.e. 38.3% of the algal taxons participated ! In addition the *Tetraëdrons* merit particular attention with their 8 taxons. From the two Planctomyces *Planctomyces Békefii* was frequent.

In the sample from the bottom taken out from the place of the algal bloom we have identified 115 algal taxons, that is 34 more than in the algal bloom developing above the sampling. The presence of these various and numerous organisms proves that, in spite of the light-reducing effect of the algal bloom, the conditions were favourable for the bottom vegetation. The distribution of the 115 taxons was: Cyanophyta 19 = 16.5%, Euglenophyta 1 = 0.9%, Chlorophyta 93 = 80.9% — including 91 Chlorophyceae, Chlorococcales (79.2%), and 2 Conjugatophyceae (1.7%) — Caulobacteriales (Planctomyces) 2 = 1.7%. Also here, the blue-green and the green algae are represented by the most species. Variability of the green algae is especially conspicuous: their 91 taxons amount to 79.2% of the taxons ! Among the 19 sorts of blue-green algae

the thread-like forms occurred in a greater number than in the algal bloom; as to the unicellular forms, the same taxons occurred as in the algal bloom. From the green algae species of *Scenedesmus*, *Tetraëdron* and *Ankistrodesmus* were still more variable. *Scenedesmus* is represented by 52 taxons, i.e. 45% of all the algal taxons. At the bottom the number of the *Scenedesmus* taxons overpassed by 21 that of the algal bloom. The *Tetraëdrons* too were more variable (12 taxons). The two sorts of Planctomyces occurred also here, but much less frequently (Figs 1, 2).

The vegetation of the bottom was therefore much more variable than that of the algal bloom. This is not surprising because in mass vegetation the number of taxons is generally low. The 81 taxons of the algal bloom are never-

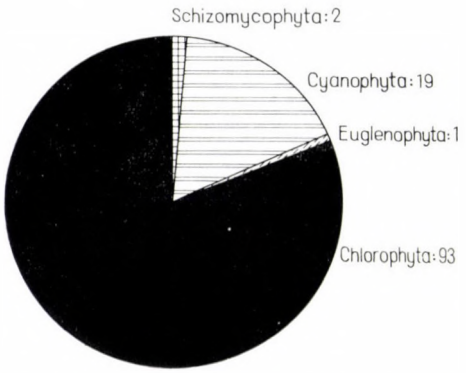

Fig. 2. Qualitative diagram of the bottom plants in the algal bloom of Hanoi. The numbers beside the names of the phyla indicate the taxon number

theless very conspicuous when compared with the algal blooms observed in Hungary in biotops of similar type. Certain backwaters, or the fish-ponds dunged sistematically can be regarded as biotops similarly contaminated as the Viet-Namese lake. In the 11 well developed algal blooms observed by us in the Nagyfa-backwater of the Tisza, for example, the number of algal taxons fluctuated between 1 and 20. In the algal bloom observed in the fish-pond No. 2, of the fishbreeding in Zimona we identified 18 sorts of organisms in June, 26 sorts in September. In the algal bloom observed in an oxidative lake of Alaska in the months June to December we found 5 to 8 alga-sorts. It seems that algal blooms of the tropical or subtropical biotops are much richer in species than the mass-vegetations northwards from them. In the Viet-Namese lake the organisms of the Euglenophyta and the Conjugatophyceae are not significant. Qualitatively, in both biotops the green algae, followed by the blue-green algae, are most significant; 96.6% and 97.4% of taxons observed at the water surface and at the bottom, respectively, belong to these two phyla.

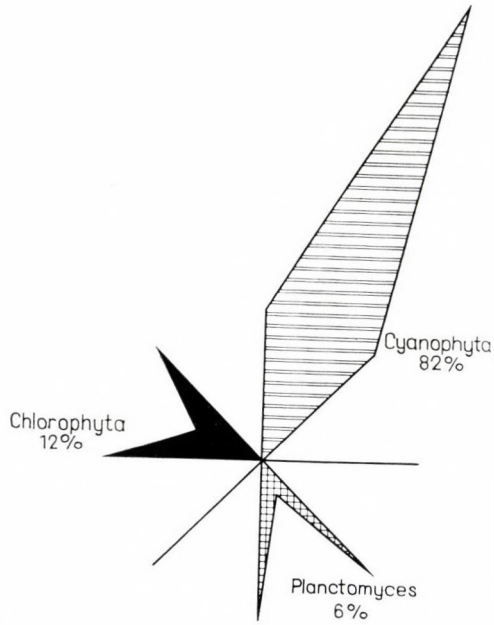

Fig. 3. Quantitative diagram of the algal bloom in Hanoi

Quantitative conditions

As to the quantitative conditions of the algal bloom, 82% of the algae were Cyanophyta, 12% Chlorophyta and 6% Planctomyces. The algal bloom contained practically no diatoms. The presence of Planctomyces, especially that of *Planctomyces Békefii* is conspicuous.

Among the bottom plants still more blue-green algae were found: the number of Cyanophyta totalled 92% of all the organisms. These are followed by the green algae (6.8%). The remaining 1.2% is represented by the diatoms of the bottom. On the bottom Planctomyces occur rarely. Consequently, the algal bloom is a definitely cyanophytic mass vegetation and such is, all the more, the biocoenosis developed at the bottom. The significant difference is due to the Planctomyces and the diatoms respectively. Though the number of individuals of the Chlorococcales is much more less in both biotops than that of the Cyanophyta, however, their variability is the greatest in both places (Figs 3, 4).

No definite line of demarcation can be found between the two biotops. The algal bloom was developed above the bottom and the plants of the bottom can easily come near to the surface by means of waves, convectional streamings and the movements of larger animals of the water. Nevertheless the two biotops can easily be distinguished their biocoenoses being not the same. Apart from the

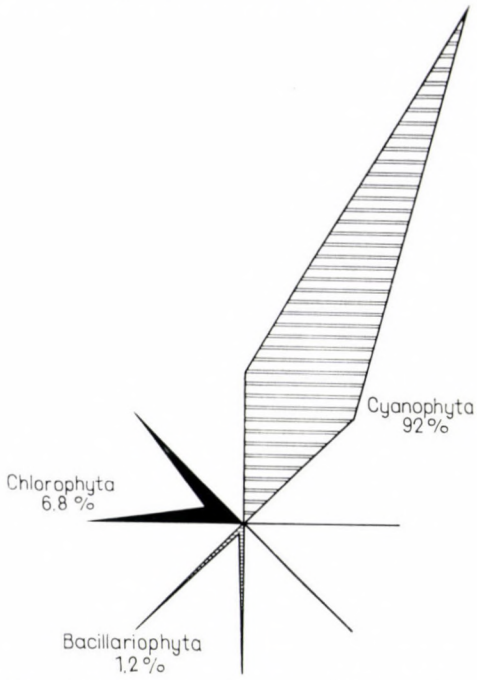

Fig. 4. Quantitative diagram of the benthos



Fig. 5. Hanoi, the Little Lake at the place of collection. Photo: Tamás Pócs

Bacillariophyceae, we have identified 130 taxons on the whole in the two samples. From all these, there are only 81 from the water surface whilst 115 from the bottom. The difference in number already shows the difference between the two biotops. The number of the common taxons is 66, i.e., 51% of all the taxons. Fifteen taxons were found in the algal bloom only, while 49 only in the benthos. These numbers further support distinctness of the two biotops.

Differences show themselves also within groups. Thus, the algae of the ordo Chlorococcales of the Cyanophyta were more variable in the algal bloom, thread-like bluegreen algae at the bottom. Also for the Chlorophyceae the bottom is more favourable, as shown by the greater number of taxons.

New taxons

In the course of the present examinations several organisms have proved to be undescribed.

These are the following:

Chlorophyceae:

Pediastrum tetras (EHR.)RALFS var. *cavum* HORTOB.
Scenedesmus polyglobulus HORTOB. f. *granulatus* HORTOB.
Scenedesmus armatus CHOD. var. *boglariensis* HORTOB. f. *crassicaudatus* HORTOB.
Scenedesmus armatus CHOD. var. *crassi-heterocaudatus* HORTOB.
Scenedesmus dispar BRÉB. var. *costatus* HORTOB. et NÉMETH f. *crassispinous* HORTOB.
Scenedesmus dispar BRÉB. var. *costato-granulatus* HORTOB. f. *duplex* HORTOB.
Scenedesmus dispar BRÉB. var. *scutatus* HORTOB.
Scenedesmus Kozmae HORTOB.
Scenedesmus quadricauda (TURP.) BRÉB. var. *mirificus* (HORTOB.) UHERKOV. f. *deflexus* HORTOB.
Scenedesmus maximus (W. et W.) CHOD. f. *granulatus* HORTOB.
Scenedesmus ellipsoideus CHOD. var. *hanoiensis* HORTOB.
Scenedesmus opoliensis RICHT. f. *deflexus* HORTOB.
Scenedesmus opoliensis RICHT. var. *bravicaudatus* HORTOB.
Scenedesmus opoliensis RICHT. f. *heterogranulatus* HORTOB.
Scenedesmus opoliensis RICHT. var. *lenticulatus* HORTOB.
Scenedesmus opoliensis RICHT. var. *crassicaudato-granulatus* HORTOB.
Scenedesmus opoliensis RICHT. var. *cornuto-granulatus* HORTOB.
Scenedesmus carinatus (LEMM.) CHOD. var. *hanoiensis* HORTOB.
Scenedesmus decorus HORTOB. var. *bicaudato-granulatus* (HORTOB.) UHERKOV. f. *crassispinosus* HORTOB.
Scenedesmus spinosus CHOD. f. *crassi-heterocaudatus* HORTOB.
Scenedesmus spinosus CHOD. var. *bicaudatus* HORTOB. f. *crassicaudatus* HORTOB.
Scenedesmus spinosus CHOD. var. *bicaudatus* HORTOB. f. *hanoiensis* HORTOB.
Scenedesmus pseudoarmatus HORTOB.
Scenedesmus pseudoarmatus HORTOB. var. *bicaudatus* HORTOB.
Scenedesmus Pócsii HORTOB.
Scenedesmus Pócsii HORTOB. var. *bicaudatus* HORTOB.
Scenedesmus pseudoopoliensis HORTOB.
Scenedesmus Soói HORTOB. f. *granulatus* HORTOB.
Scenedesmus columnatus HORTOB. var. *tropicus* HORTOB.
Scenedesmus columnatus HORTOB. var. *sexangulus* HORTOB.
Scenedesmus columnatus HORTOB. var. *bicaudatus* HORTOB. f. *hanoiensis* HORTOB.
Tetrastrum heteracanthum (NORDST.) CHOD. f. *crassispinum* HORTOB.
Tetrastrum staurogeniaeforme (SCHROED.) LEMM. f. *crassispinum* HORTOB.

The distribution of the new taxons: species 4, varietas 13 and forma 16. Out of the 33 new organisms 30 belong to the genus Scenedesmus; they are all

green algae belonging to the ordo Chlorococcales. The description of the new taxons as well as their diagnostics will be published soon.

Other results

The presence of several organisms deserves attention. Thus, the *Spirulina abbreviata* f. *minor* described from Hungary; the rare *Ankistrodesmus falcula; Chodatella balatonica* described from the Balaton; *Miracantha minutissima.* Numerous *Scenedesmus* species described from Hungary are living in Hanoi too, e.g., *Scenedesmus acuminatus* f. *globosus*, *S. denticulatus* f. *granulatus*, *S. graciosus*, *S. splendidus*, *S. dispar* var. *costato-granulatus*, *S. quadricauda* f. *crassiaculeatus*, *S. quadricauda* var. *obtusospinosus*, *S. quadricauda* var. *crassicaudatus* and his form f. *granulatus*, *S. quadricauda* var. *cornuto-granulatus*, *S. opoliensis* var. *bicaudatus*, *S. opoliensis* f. *granulatus*, *S. carinatus* f. *granulatus*, *S. spinosus* var. *crassispinosus*, *S. spinosus* var. *bicaudatus*. Among *Tetraëdrons Tetraëdron caudatum* var. *longispinum* is very characteristic of the biotops. *Tetraëdron minimum* var. *apiculato-scrobiculatum* f. *polypapillatum* described from the fish-ponds of Buzsák (Hungary) are found also in India as well as *T. pentaëdricum* f. *granulatum* is very characteristic of the benthos.

The parallel morphoses occurring regularly also in Viet-Nam are especially well demonstrated by the *Scenedesmus* species of the biotops *(S. acuminatus*, *S. armatus*, *S. dispar*, *S. quadricauda*, *S. opoliensis*, *S. carinatus*, *S. spinosus* and the forms having a columned base).

As mentioned in a preceding report [11], the fish-ponds of Hungary are very rich in *Scenedesmus* species. These biotops fed from natural currents are systematically supplied with food and dunged. We have described 183 *Scenedesmus* taxons from the fish-ponds of Buzsák. In the three waterbasins situated by the side of the stream Jamuna and the two biotops of Hanoi we identified 71 and 58 taxons of *Scenedesmus*, respectively. Considering that the investigations of Buzsák lasted throughout the year, that of India three months, whereas from Viet-Nam samples were taken on a single occasion, the material of Viet-Nam may be regarded at least as rich as the former ones. In the genera *Pediastrum*, *Scenedesmus*, *Tetraëdron* and *Tetrastrum* we observed several interesting teratoms. These will be described in the papers elsewhere [12, 13].

It is conspicuous in Hanoi that from the spiny forms of the ordo Chlorococcales that of thin spines are rare, whereas the literature considers these as more frequent. It is so especially among the *Scenedesmus*, but it is frequent among the *Tetraëdron* and *Tetrastrum* species too. A similar situation can be found in the case of the Indian *Scenedesmus;* also there, the taxons of strong spines dominate, however, the obtuse spine ending is not so frequent as in Hanoi. It seems that towards the tropics the spines, ribs, granules and protu-

berances of the membrane become more and more stronger: like higher plants, the algae from the tropics are more developed. The explication is to be searched in environmental conditions.

Organisms of the algal bloom and the lake bottom

	Algal bloom	Bottom
Cyanophyta		
Chlorococcales		
1. *Aphanocapsa elachista* var. *conferta*	+	
2. *Aphanocapsa elachista* var. *planctonica*	+	+
3. *Coelosphaerium confertum*	+	+
4. *Chloronostoc abbreviatum*	+	+
5. *Chiorococcus varius ?*		+
6. *Gloeocapsa granosa*	+	+
7. *Merismopedia glauca* f. *insignis*	+	+
8. *Merismopedia punctata*	+	+
9. *Merismopedia tenuissima*	+	+
10. *Microcystis parasitica*	+	+
11. *Microcystis robusta*	+	
12. *Pelodictyon clathriforme*	+	
13. *Synechocystis crassa*	+	+
	12	10
Hormogonales		
14. *Anabaena spiroides* f. *minima*	+	
15. *Lyngbya bipunctata*		+
16. *Lyngbya circumcreta*	+	+
17. *Lyngbya Lagerheimii*		+
18. *Lyngbya perelegans*	+	
19. *Oscillatoria Agardhii*		+
20. *Oscillatoria irrigua*		+
21. *Romeria elegans*		+
22. *Spirulina abbreviata* f. *minor*	+	+
23. *Spirulina laxissima* f. *major*	+	+
24. *Spirulina subtilissima*	+	+
	6	9
sum total of Cyanophyta:	*18*	*19*
Euglenophyta		
25. *Astasia* sp.	+	+
	1	1
sum total of Euglenophyta:	*1*	*1*
Chlorophyta		
Chlorophyceae		
Chlorococcales		
26. *Ankistrodesmus convolutus*	+	+
27. *Ankistrodesmus convolutus* var. *minutum*		+
28. *Ankistrodesmus falcatus* f. *Marthae*	+	+
29. *Ankistrodesmus falcatus* var. *spirilliformis*	+	+
30. *Ankistrodesmus falcula*	+	+
31. *Schroederia setigera*	+	+

	Algal bloom	Bottom
32. *Schroederia spiralis*	+	+
33. *Chodatella balatonica*	+	+
34. *Chodatella ciliata*	+	
35. *Coelastrum cubicum*	+	+
36. *Dictyosphaerium pulchellum* var. *ovatum*	+	+
37. *Franceia polychaeta ?*		+
38. *Gloeoactinum limneticum*	+	+
39. *Golenkinia radiata*	+	+
40. *Micracantha minutissima*		+
41. *Nephrochlamys allantoidea*	+	+
42. *Nephrocytium lunatum*		+
43. *Oocystis crassa* var. *Marssonii*	+	+
44. *Oocystis parva*	+	+
45. *Pediastrum clathratum* var. *asperum*	+	+
46. *Pediastrum clathratum* var. *punctatum*	+	+
47. *Pediastrum tetras* var. *cavum*		+
48. *Pediastrum tetras* var. *tetraodon*		+
49. *Siderocelis ornata*	+	+
50. *Tetraëdron caudatum*		+
51. *Tetraëdron caudatum* var. *longispinum*	+	+
52. *Tetraëdron caudatum* var. *punctatum*	+	+
53. *Tetraëdron hastatum*	+	
54. *Tetraëdron hastatum* var. *palatinum*	+	
55. *Tetraëdron minimum* var. *apiculato-scrobiculatum*	+	+
56. *Tetraëdron minimum* var. *apiculato-scrobiculatum f. polypapillatum*		+
57. *Tetraëdron muticum* f. *punctulatum*	+	+
58. *Tetraëdron pentaëdricum*	+	+
59. *Tetraëdron pentaëdricum* f. *granulatum*		+
60. *Tetraëdron regulare* var. *incus*		+
61. *Tetraëdron trigonum* var. *papilliferum*		+
62. *Tetraëdron trigonum* var. *subtetraëdricum*		+
63. *Tetraëdron trilobatum*	+	+
64. *Tetrastrum heteracanthum*	+	+
65. *Tetrastrum heteracanthum* f. *crassispinum*		+
66. *Tetrastrum staurogeniaeforme*		+
67. *Tetrastrum staurogeniaeforme* f. *crassispinum*		+
68. *Treubaria triappendiculata*		+
69. *Scenedesmus ecornis*		+
70. *Scenedesmus acuminatus* f. *globosus*	+	+
71. *Scenedesmus acuminatus* var. *elongatus*	+	+
72. *Scenedesmus acuminatus* f. *tortuosus*	+	
73. *Scenedesmus tetradesmiformis*	+	+
74. *Scenedesmus wisconsinensis*	+	+
75. *Scenedesmus polyglobulus* f. *granulatus*	+	+
76. *Scenedesmus denticulatus* f. *granulatus*	+	+
77. *Scenedesmus intermedius*		+
78. *Scenedesmus graciosus*	+	
79. *Scenedesmus splendidus*	+	
80. *Scenedesmus Lefevrii*		+
81. *Scenedesmus armatus*	+	
82. *Scenedesmus armatus* var. *boglariensis* f. *crassicaudatus*	+	+
83. *Scenedesmus armatus* var. *crassi-heterocaudatus*	+	+
84. *Scenedesmus dispar* var. *costatus* f. *crassispinosus*		+
85. *Scenedesmus dispar* var. *costato-granulatus*	+	+
86. *Scenedesmus dispar* var. *costato-granulatus* f. *duplex*	+	+
87. *Scenedesmus dispar* var. *scutatus*		+
88. *Scenedesmus Kozmae*		+
89. *Scenedesmus quadricauda* f. *crassiaculeatus*	+	+
90. *Scenedesmus quadricauda* var. *obtusospinosus*	+	+
91. *Scenedesmus quadricauda* var. *crassicaudatus*		+

	Algal bloom	Bottom
92. *Scenedesmus quadricauda* var. *crassicaudatus* f. *granulatus*		+
93. *Scenedesmus quadricauda* var. *cornuto-granulatus*		+
94. *Scenedesmus quadricauda* var. *mirificus* f. *deflexus*		+
95. *Scenedesmus maximus*		+
96. *Scenedesmus maximus* f. *granulatus*	+	
97. *Scenedesmus ellipsoideus* var. *hanoiensis*		+
98. *Scenedesmus opoliensis*	+	+
99. *Scenedesmus opoliensis* f. *deflexus*		+
100. *Scenedesmus opoliensis* var. *bicaudatus*	+	+
101. *Scenedesmus opoliensis* var. *brevicaudatus*		+
102. *Scenedesmus opoliensis* var. *mononensis*	+	+
103. *Scenedesmus opoliensis* f. *granulatus*	+	+
104. *Scenedesmus opoliensis* f. *heterogranulatus*	+	+
105. *Scenedesmus opoliensis* var. *lenticulatus*	+	+
106. *Scenedesmus opoliensis* var. *crassicaudato-granulatus*	+	+
107. *Scenedesmus opoliensis* var. *cornuto-granulatus*		+
108. *Scenedesmus carinatus* f. *granulatus*	+	+
109. *Scenedesmus carinatus* var. *hanoiensis*		+
110. *Scenedesmus decorus* var. *bicaudato-granulatus* f. *crassispinosus*		+
111. *Scenedesmus ornatus*	+	+
112. *Scenedesmus spinosus*	+	+
113. *Scenedesmus spinosus* var. *crassispinosus*	+	+
114. *Scenedesmus spinosus* f. *crassi-heterocaudatus*	+	+
115. *Scenedesmus spinosus* var. *bicaudatus*	+	+
116. *Scenedesmus spinosus* var. *bicaudatus* f. *crassicaudatus*	+	+
117. *Scenedesmus spinosus* var. *bicaudatus* f. *hanoiensis*		+
118. *Scenedesmus pseudoarmatus*		+
119. *Scenedesmus pseudoarmatus* var. *bicaudatus*		+
120. *Scenedesmus Pócsii*		+
121. *Scenedesmus Pócsii* var. *bicaudatus*		+
122. *Scenedesmus pseudoopoliensis*		+
123. *Scenedesmus Soói* f. *granulatus*		+
124. *Scenedesmus columnatus* var. *tropicus*		+
125. *Scenedesmus columnatus* var. *sexangulus*		+
126. *Scenedesmus columnatus* var. *bicaudatus* f. *hanoiensis*		+
sum total of Chlorophyceae:	*58*	*91*
Conjugatophyceae		
127. *Staurastrum perundulatum*	+	+
128. *Staurastrum* sp.	+	+
sum total of Conjugatophyceae:	*2*	*2*
Schizomycophyta		
Caulobacteriales		
129. *Planctomyces Békefii*	+	+
130. *Planctomyces guttaeformis*	+	+
sum total of Schizomycophyta:	*2*	*2*
sum total of all the groups	81	115
Cyanophyta	18=22.2%	19=16.5%
Euglenophyta	1= 1.3%	1= 0.9%
Chlorophyta	60=74.0%	93=80.9%
Chlorophyceae		
Chlorococcales 58=71.5%;	91=79.2%	
Conjugatophyceae 2= 2.5%;	2= 1.7%	
Schizomycophyta, Caulobacteriales	2= 2.5%	2= 1.7%
	81=100%	115=100 %

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ÜBER EINE WASSERBLÜTE IN VIETNAM

Bei Hanoi, in einem schon seit langem abgeschnürten toten Arm des Roten Flusses mit verunreinigtem Wasser hatte sich im Januar 1966 nach einer Regenfront im Winter-Monsumklima eine hellgrüne Wasserblüte herausgebildet. Die Wassertemperatur betrug während der Wasserblüte +13—26 °C. Die Wasserblüte war äußerst reich an Arten und neuen Taxonen. Es nahmen daran insgesamt 81 Taxone teil. Qualitativ waren die Blau- und Grünalgen vorherrschend. Die Arten der Gattung *Scenedesmus* zeichneten sich durch besonders große Vielfalt der Form aus. Im Benthos lebten unter der Wasserblüte noch vielerlei Pflanzen, insgesamt 115 Taxone. Auch hier gehörten die meisten Taxone den Blau- und Grünalgen an und die *Scenedesmus*-Arten waren noch mannigfaltiger. Mengenmäßig dominierten in beiden Biotopen die Blaualgen, mit 82% bzw. 92%. Zwischen den beiden Biotopen ließ sich keine scharfe Grenze ziehen, doch waren die Phytozönosen nicht identisch. 66 Taxone, d.h. 51%

aller Taxone kamen in beiden Biotopen vor. 33 Taxone waren in der Wissenschaft unbekannt, 30 dieser neuen Taxone gehörten zu der Gattung *Scenedesmus*. Die letzteren liefern besonders schöne Beispiele der gesetzmäßig auftretenden parallelen Morphosen. In Hanoi lassen sich unter den stacheltragenden Taxonen der Gattung Chlorococcales auffallend viele mit dicken Stacheln nachweisen. Es scheint, daß in Richtung zu den Tropen die Stacheln, Rippen und Körnchen zunehmend stärker werden.

ЦВЕТЕНИЕ ВОДЫ ВО ВЬЕТНАМЕ

В Ханое, в уже давно отшнуровавшемся мертвом рукаве реки Хонг-Ха (Красная река) с загрязненной водой в январе 1966 года после дождевого фронта в зимнем муссонном климате развилось яркозеленое цветение воды. Температура воды составила + 13—26° С. Цветение воды было исключительно богато видами и новыми таксонами. В нем участвовал 81 таксон. Качественно господствовали синезеленые и зеленые водоросли. Особенно виды *Scenedesmus* отличались богатством форм. В бентосе, под цветением воды жило еще множество растений: 115 таксонов. Большинство таксонов бентоса относилось также к синезеленым и зеленым водорослям, а виды рода Scenedesmus были еще более разнообразными. Количественно в обоих биотопах господствовали синезеленые водоросли, составляя 82% и 92% всей популяции. Между двумя биотопами нельзя провести резкой границы, но фитобиоценозы все же не были идентичными. Число таксонов, живущих в обоих биотопах, составляет 66, это 51% всех таксонов. 33 таксона были новыми для науки, 30 из них относятся к роду Chlorococcales. Последние особенно наглядно показывают закономерно появляющиеся параллельные морфозы. В Ханое среди игольчатых таксонов рода Chlorococcales наблюдались очень многие виды с толстыми игольками. Кажется, что в направлении к тропическим странам иголки, ребра и зернышки становятся все более выраженными.

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STIMULATION OF THE PROCESSES OF SOMATIC EMBRYOGENESIS IN SOME PORIFERA AND COELENTERATA

II. PROVOCATION OF SOMATIC EMBRYOGENESIS IN LAOMEDEA FLEXUOSA HINKS BY COLCHICINE AND MECHANICAL INJURIES

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Synopsis

Colchicine (0.001% and 0.01% concentrations) disturbing normal regularities of the cell-systems provokes somatic embryogenesis in *Laomedea flexuosa* HINKS; its various forms depend on the peculiarities of asexual reproduction characteristic of the species of Coelenterata. Formation of new hydrants or hydroriza-like cilia was observed. Somatic embryogenesis can be provoked by disturbing mechanically the integrity of the cenosark. The fragments (about 1 mm in diameter) cut out of the stolon of *L. flexuosa* developed into one, two or even more polyps.

Introduction

In view of the well-known influence of colchicine on cells and tissues, it is probable that in some concentrations colchicine inhibits the processes of regeneration, and stimulates or rather provokes those of somatic embryogenesis. Yet, one should keep in mind that cells react differently to the action of similar doses of injurious agents at different stages of their ontogeny, i.e., of their "mitotic cycle". In normal "architectonics" of tissues the correlation between cells at different stages of their ontogeny cannot be chaotic. On the other hand, cells at different stages of their life cycle react differently to the action of the same injurious agent. That is why injurious agents, which can kill some cells, appear to be neutral to, or even stimulate others, inevitably disturb the normal integration of cell systems [5].

There have been no special works devoted to the study of Coelenterata on the line of these hypotheses. But the works of HAM and EAKIN [1, 2] present some interest in that they speak of colchicine inhibiting the regeneration in hydra. According to their own observations, colchicine, if added to the culture media four hours after operation (the cutting out of a part of the body), does not affect regeneration.

HAMMET and REINMANN [3] affirm that placing the colonies of *Obelia geniculata* in sea water with some di-benzanthracene dissolved in benzene, added to it, stimulates new hydrant growth.

Material and method

TOKIN has carried out 7 series of experiments on more than 700 colonies of *Laomedea flexuosa* HINKS, inhabitant of the Barents Sea. Most experiments were carried out in the following way. Ten or more colonies, with all their hydrants cut off, were placed in a sea-water solution of colchicine of various concentrations (0.001%, 0.01%, 0.05%) for 18, 46 or 48 hours. The same number of colonies, with all their hydrants cut off, were placed in stagnant sea water of the same temperature.

The process of somatic embryogenesis, i.e., the formation of new hydrants, and the rate of their growth were closely observed. Their emergence was carefully registered. The three developmental stages of the hydrant are shown in Fig. 1.

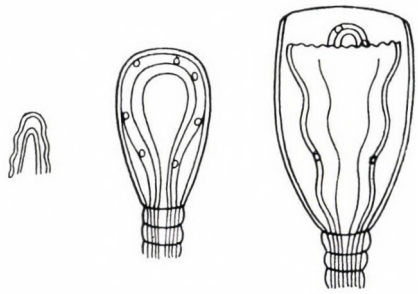

Fig. 1. Laomedea flexuosa. Scheme of the stages of hydrant development: I — the 1st stage, II — the 2nd stage, III — the 3rd stage

Results

Colchicine is highly toxic in a concentration of 0.05%: colonies of *Laomedea*, in good condition and non-traumatized, perished in it after 1.5—2 days. But in a 0.01% colchicine solution they live without any visible harm for at least 3 days. Colchicine in 0.001% or 0.01 solution provokes somatic embryogenesis. The records of a typical experiment are as follows.

Ten control colonies with their hydrants removed, are placed in the stagnant sea water. Experimental colonies (10 of each variant) are placed in colchicine solution for 18 hours; then these as well as the control ones are put in running sea water. The results registered after 24 hours are shown in Table 1.

Table 1

Effect of a colchicine solution on somatic embryogenesis

Concentration of colchicine per cent	No bud at the site of injury per cent	Buds at stage I per cent	Hydrants at stage II per cent	Hydrants at stage III per cent
0	51.5	31.1	17.4	0
0.001	21.5	57.8	14.5	6.2
0.01	20.9	53.6	20.9	4.6
0.05	38.5	59.0	2.5	0

These data show that colchicine in the indicated concentrations can stimulate somatic embryogenesis (Table 1). In all cases there are much more buds at the first stage in colchicinized colonies than in the control ones. Besides stimulating the onset of somatic embryogenesis, it seems that colchicine increases the rate of development: 0.001% and 0.01% concentrations gave fully developed hydrants in 6.2% and 4.6% respectively, while there were none in the control colonies. Nevertheless, this fact may be due to colchicine provoking the initiation of buds, while the rate of their growth may remain the same as in the control specimens. Colchicine in a 0.05% concentration provokes an energetic onset of bud formation, i.e., of somatic embryogenesis, but then the development stops, hydrants fail to grow, and the animals invariably die.

Table 2

Influence of colchicine on somatic embryogenesis

Concentration of colchicine per cent	No buds formed per cent	Buds at stage I per cent	Hydrants at stage II per cent	Hydrants at stage III per cent
0	28	52.4	15.4	4.2
0.01	14.3	17.1	52.8	15.8
0.001	27.3	24.7	42.1	5.9

Even a lower concentration — 0.01% — proved lethal for some specimens. These died 60 hours or more after the colchicine treatment. It is to be noted that they have a higher per cent of well formed hydrants by that time than the control ones.

There is no doubt whatever that colchicine stimulates somatic embryogenesis. Table 2 shows the results of another series of experiments in which the colonies, after the amputation of all their hydrants, were placed in a colchicine solution for 46 hours, instead of 18 hours.

Remarkable is the high incidence of well-developed hydrants (15.8%) in the "colchicinized" colonies, especially as compared with the control ones (4.2%): after 75 hours the former had 46.2% of well-developed hydrants, while the latter had only 5.0%.

Microscopic investigations showed the deep disturbances which colchicine produces in the normal structure of cell systems, the cenosark of the colony, losing its continuity, and so the integrity of the whole colony is destroyed. This creates a basis for the above-described growth of hydrants in the sites of those cut off and for other phenomena of somatic embryogenesis. Occasionally new polyps grow at quite unusual sites, e.g., an additional polyp is formed on the pedicle of the hydrant and it "burst" through the pedal perisark, along with the new polyp growing in place of the one cut off.

Such phenomena should not be termed "monstrosities". They are the result of a disturbance of naturally but slightly integrated colonies of hydroid polyps, in which normal regulation of the life of cell systems has become lost. In this condition some somatic cells and their aggregates are in a morphological isolation, devoid of the formative influence of the whole organism and may, as



Fig. 2a. Somatic embryogenesis in *Laomedea flexuosa:* development of hydrant on the area of cutting out from hydrorhiza (photo)

is shown by experiments on many organisms [5], become analogous to sexual cells and develop into organisms. The forms of manifestation of somatic embryogenesis are various, depending on the ways and the peculiarities of the asexual reproduction characteristic of the species. KOROTKOVA proved this fact in her studies on sponges and substantiated it theoretically [4].

Here are some examples of "monstrosities" in the formative processes induced by colchicine. A colony of *Laomedea*, after 3 days in a 0.01% colchicine solution, developed three long "lashes" covered with perisark almost throughout their whole length in the places of the cut. Being placed in running sea water, the colony survived for nine days and formed 4 quite normal hydrants

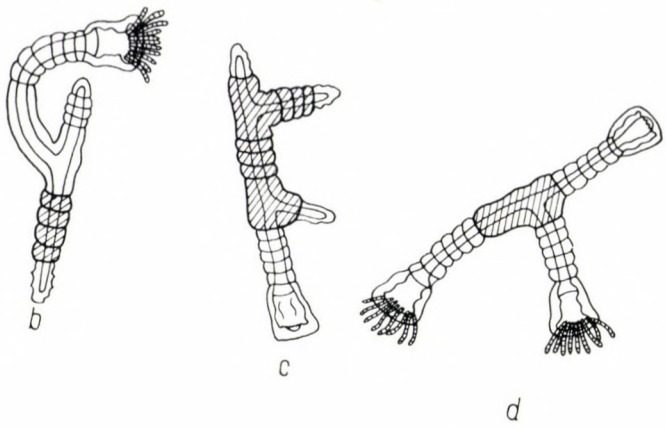



Fig. 2b, c, d, e. Somatic embryogenesis in *Laomedea flexuosa.* Different cases of somatic embryogenesis (the development of hydrants on the fragments of stolon; *e* = photo)

and 2 buds in stage I of development, the whole colony and the "lashes" attaining a length of about 4.5 cm and 1 cm, respectively. Its cenosark was continuous, but irregular in thickness, and was covered by a smooth perisark of a normal structure, except at the distal end. Then a bud appeared on one of the "lashes". The lashes went on growing and after two days one of them was 1.5 cm long, its end was attached to the glass, a phenomenon characteristic of the hydrorhiza. The other "lash" developed hydrants at stage II and stage III. Such "lashes" are certainly potential hydrorhizas (Fig. 2a).

In another case a hydrant bud appeared at the site where the stolon was cut off the hydrorhiza, and then developed into a normal hydrant (Fig. 2b).

As mentioned above, REINMANN and HAMMET succeeded in provoking hydrant formation in *Obelia geniculata* by applying cancerogenic substances. TOKIN, however, using 9.10-dimethyl 12 benzanthracene and urethane failed to reproduce this result in hydroid polyps.

"Lash" formation was also observed by KOROTKOVA and TOKIN, who used ^{90}Sr and ^{90}Y in studying the influence of irradiation on hydroid polyps. These and similar data are of interest for they show these hydrorhiza-like "lashes" to be a kind of asexual reproduction, this being confirmed by some literary data.

Can a mechanically induced disorganization of Laomedea provoke somatic embryogenesis?

When the hydrants of ten colonies are cut off with a scalpel and then every stolon is pressed with a blunt side of an eye scalpel at ten sites, the cenosark is, naturally, severely disturbed at the sites of pressure, and the colonies cannot preserve their normal integration. The control colonies have their hydrants cut off, but their cenosark is not disturbed. Both groups, the experimental and the control ones, are put in the same condition in running sea water. In these experiments the rough mechanical disintegration stimulated somatic embryogenesis (Table 3).

Table 3

Stimulation of somatic embryogenesis by mechanical trauma
(Two days after the beginning of the experiment)

Colonies	No buds at the site of cutting per cent	Hydrants at stage I per cent	Hydrants at stage II per cent	Hydrants at stage III per cent
Control	59	40	1	1
Experimental	37	60	2	1

Morphogenetic observations showed the formation of hydrorhiza-like lashes in some experimental colonies in the sites of cutting, a phenomenon having taken place in some of the mentioned experiments as well. One more fact is of interest: the cenosark wounded by a mechanical trauma restores its integrity in two days, so that no trace of the trauma remains to be discerned.

In another extensive series of experiment all hydrants of a colony of *Laomedea* were cut off. Then the stolons were cut into pieces 1 mm in diameter. Three days after the operation they all showed signs of starting to form new hydrants. The variety of formative processes was striking: one piece giving one,

two, three or even four polyps. New hydrants can also develop from a piece of the stolon cut out of an interganglial part, and may grow from fragments of a ganglion or of a pedicle of the polyp (Fig. 2e).

Observation of such "monstrosities" during 10—15 days shows that they are in a state of normal life and that they are separate organisms. Yet in no case has the growth of any stolon-like formation, of a hydrorhiza or of a whole colony been traced among them. Neither do they attach to the glass or to any other object around them.

Discussion

The results of the last series of experiments as well as those mentioned earlier suggest that in *Laomedea* there is no need of a colony or of any particular integration of it as a whole to induce embryogenesis to start and to form quite normal new hydrants, as in asexual reproduction. The experiments show that new individual polyps grow from fragments of a stolon and of cell material of a cenosark isolated from the organism. This is in accordance with the theory of somatic embryogenesis developed by TOKIN [5] and is confirmed by other data obtained in his laboratory in experiments on Coelenterata. The data obtained by TOKIN, ERITHCEVA and KALININA [6] have proved that *Laomedea* has but a very poor regenerative ability, but, like other Coelenterata, possesses a very high capacity for somatic embryogenesis, the latter being easily induced by various stimuli, like punctured burns, which disturb the normal integrity of these poorly integrated organisms.

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DIE STIMULATION DER SOMATISCHEN EMBRYOGENESENPROZESSE BEI EINIGEN PORIFEREN UND COELENTERATEN

II. Provokation der somatischen Embryogenese an *Laomedea flexuosa* HINKS mit Colchicin und mechanischer Läsion

Bei *Laomedea flexuosa* HINKS stört Colchicin (in 0,001%- und 0.01%iger Konzentration) die normale Regelmäßigkeit der Zellsysteme und provoziert die somatische Embryogenese. Ihre verschiedenen Formen, die von den für Coelenteraten kennzeichnenden Besonderheiten der ungeschlechtlichen Fortpflanzung abhängen, werden besprochen. Die Entstehung neuer

Hydranten oder hydrorisaartiger Cilien wurde beobachtet. Somatische Embryogenese kann durch mechanische Störung der Integrität des Cœnosarks provoziert werden. Die aus dem Stolon von *L. flexuosa* herausgeschnittenen Fragmente (von etwa 1 mm Querschnitt) entwickelten sich zu einem, zu zwei oder sogar zu mehreren Polypen.

СТИМУЛЯЦИЯ ПРОЦЕССОВ СОМАТИЧЕСКОГО ЭМБРИОГЕНЕЗА У НЕКОТОРЫХ PORIFERA И COELENTERATA

II. Провоцирование соматического эмбриогенеза у *Laomedea flexuosa* Hinks воздействием колхицина и механическими травмами

Колхицин (в концентрации 0,001% и 0,01%), нарушая нормальные закономерности клеточных систем, провоцирует соматический эмбриогенез у *Laomedea flexuosa* Hinks. Вариация форм соматического эмбриогенеза зависит от особенностей бесполого размножения, характеризующих данный вид кишечнополостных. Наблюдается формирование добавочных гидрантов или гидроризоподобных «плетей». Соматический эмбриогенез можно вызвать, нарушая нормальную интеграцию ценосарка механическими травмами. На фрагментах (около 1 мм в диаметре), вырезанных из столона *L. flexuosa*, развивается один или несколько полипов.

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HISTOCHEMICAL STUDY OF FOLLICULAR ATRESIA IN THE AMPHIBIAN OVARY

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Synopsis

Follicular atresia and problems of interstitial gland cells in the ovary of toad *(Bufo stomaticus)* have been studied with histochemical techniques. Atresia affects follicles of all sizes, but the atresia of mature follicles is very common during winter. Details of morphological and histochemical changes in the ooplasm and egg envelops are described. During atresia of large previtellogenic follicles, some cells of thecal origin are hypertrophied and filled with lipid droplets consisting first of phospholipids and then of phospholipids and triglycerides. They constitute the interstitial gland cells which during winter store cholesterol or its esters, in addition to other lipids. The oocyte, zona pellucida and granulosa cells finally regress and disappear; the latter play an important role in the digestion and removal of yolk elements from the degenerating mature follicles which do not share any cytochemical feature with mammalian corpus luteum. The histochemistry of atretic follicles of toad has been compared and contrasted with that in reptiles and mammals. The interstitial gland cells of amphibian ovary are discussed in the light of recent cytochemical studies carried out on similar cells in the ovaries of reptiles and mammals.

Introduction

Histochemical techniques have been used to determine changes in the normal growing follicle of amphibian ovary [8]. To my knowledge, there are no previous publications on the histochemistry of follicular atresia in the amphibian ovary. However, there are some reports on the histological changes of follicular atresia [3, 26]. The present study was initiated to follow the details of histochemical changes during the regression of the various components of the follicles of all sizes. The problems of interstitial cells in the amphibian ovary will also be discussed in the light of recent researches carried out on the ovaries of other vertebrates [9]. An additional objective of this paper is to compare and contrast the histochemistry of atretic follicles of amphibian ovary with those of reptiles and mammals, which have recently been described by the present author [9].

Material and method

Ovaries of sexually mature toad *(Bufo stomaticus)* were examined throughout the year. Fixing fluids used for the histochemistry of lipids included 10% formalin [25] and formaldehyde calcium with and without postchromation [1, 2]. After fixation and subsequent postchromation,

material was embedded in gelatine after BAKER [1, 2]. Frozen gelatine sections were cut at 10 μ. Sections were washed briefly in water and subjected to the histochemical methods for lipids listed below. Material fixed in weak Bouin's fluid and extracted with pyridine after BAKER [1] and coloured with Sudan black B was used as a control for lipids in general. Material was also treated with Zenker and Carnoy and embedded in paraffin. Such paraffin sections were used for the study of carbohydrates, proteins and nucleic acids.

Histochemical tests

For lipids: Sudan black B in 70% ethanol [2] for colouring lipids in general; Nile blue sulphate technique for neutral and acid lipids [4, 5]; Sudan III and IV methods for neutral fats after KAY and WHITEHEAD [25]; the gelatine-Sudan III and Sudan IV methods after GOVAN [25] for neutral lipids; the Fettrot method as described by PEARSE [25] for neutral fats; acid haematein technique together with pyridine extraction control [1] for phospholipids; Schultz method as described by GOMORI [6] and LILLIE [22] for cholesterol or its esters. Sudanophilic lipids which stained pink in Nile blue and red in red Sudan dyes were interpreted as neutral fats (triglycerides). Acid lipids (phospholipids and fatty acids), lipoproteins and proteins generally stain blue in Nile blue. Only those sudanophilic substances which stained blue in Nile blue and bluish-black in acid haematein, together with a negative reaction in the material treated with pyridine, were considered to contain phospholipids.

For carbohydrates: Periodic acid—Schiff (PAS) test together with usual controls as reported by PEARSE [25].

For proteins: Mercuric bromphenol blue technique [23]. Material fixed in weak Bouin's solution and extracted with hot pyridine is very favourable for the demonstration of protein as the possibility of staining due to lipids is eliminated.

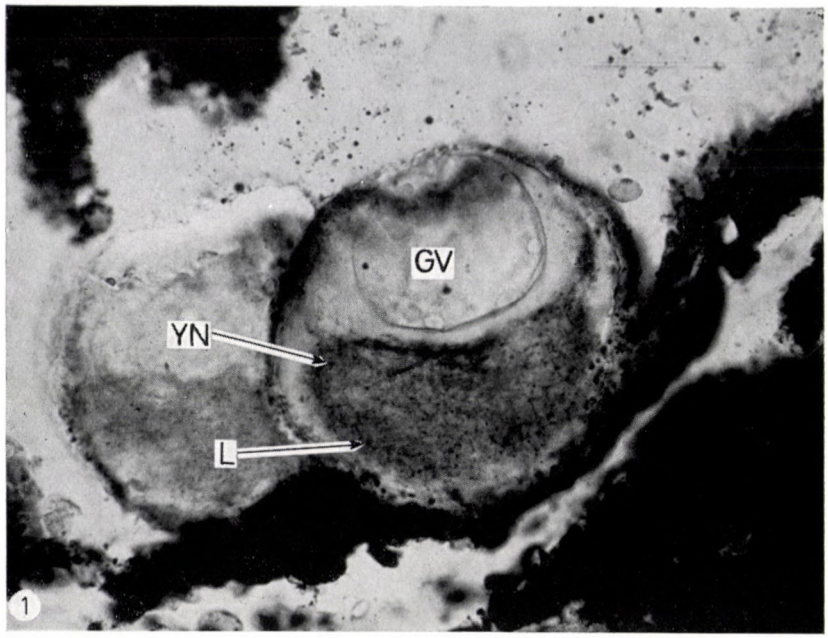
For nucleic acids: Feulgen technique as described by PEARSE [25] for DNA; pyronine methyl green technique after JORDAN and BAKER [21] together with a negative reaction in control sections treated with trichloroacetic acid [25] for RNA.

Results

Atresia may appear in follicles at any stage of development including mature follicles. The atresia in the latter is very common during winter (November to February). For the sake of convenience, the atresia of previtellogenic and vitellogenic follicles will be described separately.

Previtellogenic follicles

With the start of atresia, the previtellogenic follicles become shrunken, suggesting, at least a relative, loss of water. With the advancement of atresia, the mitochondria and the peripheral lipid bodies associated with vacuoles [8] generally disappear from view. The shrinkage is accompanied by changes in the yolk nucleus substance which has segregated into aggregations of relatively large size (Fig. 1). However, the yolk nucleus substance in the ooplasm of large previtellogenic follicles does not form such abnormal masses (Fig. 2). The ooplasm of young follicles develops some sudanophilic lipid bodies of atresia (Fig. 1). They give a moderate positive reaction in the acid haematein, which becomes negative in the material fixed in weak Bouin's fluid and extracted with hot pyridine. This indicates that the lipid bodies of atresia contain phospholipids. Some lipid bodies stain light pink in Nile blue and red in red Sudan dyes, showing some neutral fats (triglycerides), in addition to phospholipids.



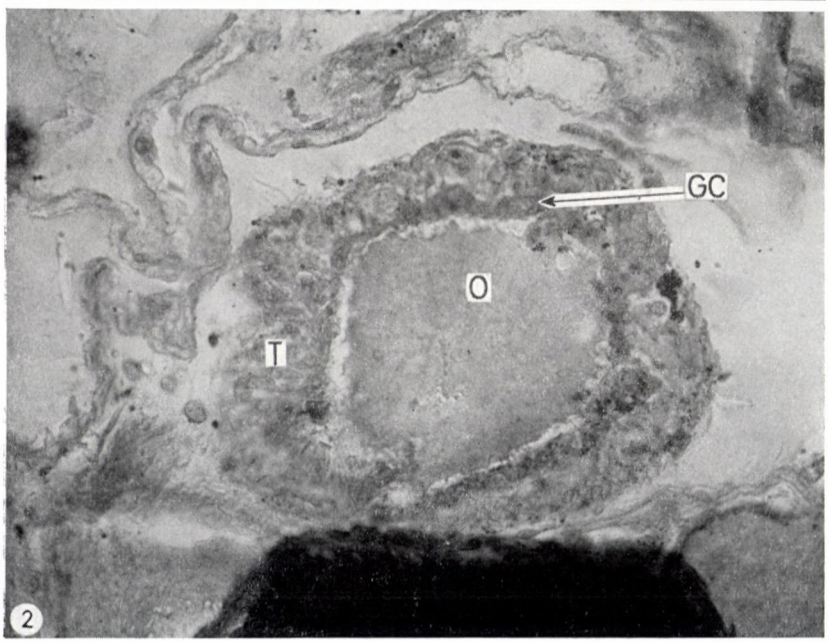


Fig. 1. Young oocyte in atresia, showing the aggregate of yolk nucleus substance (YN), sudanophilic lipid granules (L), and sudanophobic germinal vesicle (GV). Formaldehyde-calcium, postchromed in dichromate-calcium, Sudan black B. ×540

Fig. 2. Previtellogenic follicle in atresia, showing hypertrophied theca (T), and regressing oocyte (O) and granulosa cells (GC). Technique as in Fig. 1. ×300

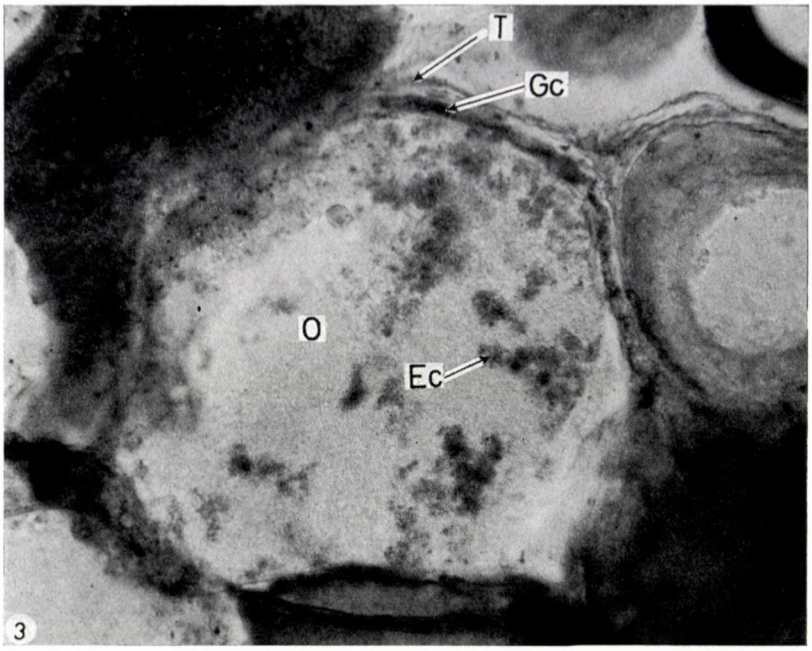
With the advancement of atresia, some epithelial cells of small size appear in the ooplasm of large previtellogenic follicles (Fig. 3). They are apparently derived from the follicle cells as judged from the close similarities in their morphology and histochemistry. These epithelial cells appear to be engaged in the digestion of ooplasmic structures as shown by their localization and distribution in the ooplasm (Fig. 3). At this stage the follicles are greatly collapsed and wrinkled.

The various components of the ooplasm and germinal vesicle in the large previtellogenic follicles, which do not show any conspicuous histochemical change demonstrable with the techniques used, are gradually resorbed, probably owing to phagocytosis by the small epithelial cells of granulosa origin. However, during the regression of large previtellogenic follicles, dramatic changes appear in the egg envelopes. The wrinkled zona pellucida, which does not show any histochemical change, continues to persist till the last stage of regression. The follicular epithelial cells (or granulosa cells) form a nodule of cells which develop sudanophilic lipid droplets. The lipid bodies stain for triglycerides and phospholipids. The cytoplasmic basophilia observed in the normal follicle cells is not seen. There is no ingrowth of connective tissue (theca) which becomes thickened and hypertrophied, owing to the contraction of the follicular wall (Fig. 2). The follicular epithelial cells are ultimately resorbed, leaving behind the elements of the original theca (Fig. 4), which form conspicuous bodies in the ovary. The blood vascularity of this nodule of hypertrophied stromal tissue is increased, in comparison to the previous stages of atresia. Some elements of stromal tissue are relatively more hypertrophied. Their cytoplasm stores increasing amounts of sudanophilic lipid droplets (Fig. 4) which consist of phospholipids in the first stage and of triglycerides and phospholipids later. However, during winter, the lipid droplets of these cells of thecal origin also accumulate cholesterol or its esters, in addition to triglycerides and phospholipids. The hypertrophied cells of stromal (thecal) origin which are filled with sudanophilic lipid droplets, are distributed in groups or patches in the nodules of ovarian stroma (Fig. 4); their lipid accumulations are not seen in summer.

Vitellogenic follicles

During winter, several large follicles full of yolk undergo degeneration. Such degenerating follicles form a prominent feature of the ovaries of *Bufo stomaticus*. For the sake of convenience, the atresic process of mature follicles will be divided into three stages based on the morphological and histochemical changes.

Stage 1. During the first stage of atresia, the atretic follicle is similar to the normal one. However, a close examination reveals some features not seen in the normal follicle of the same size. The first demonstrable change in a follicle



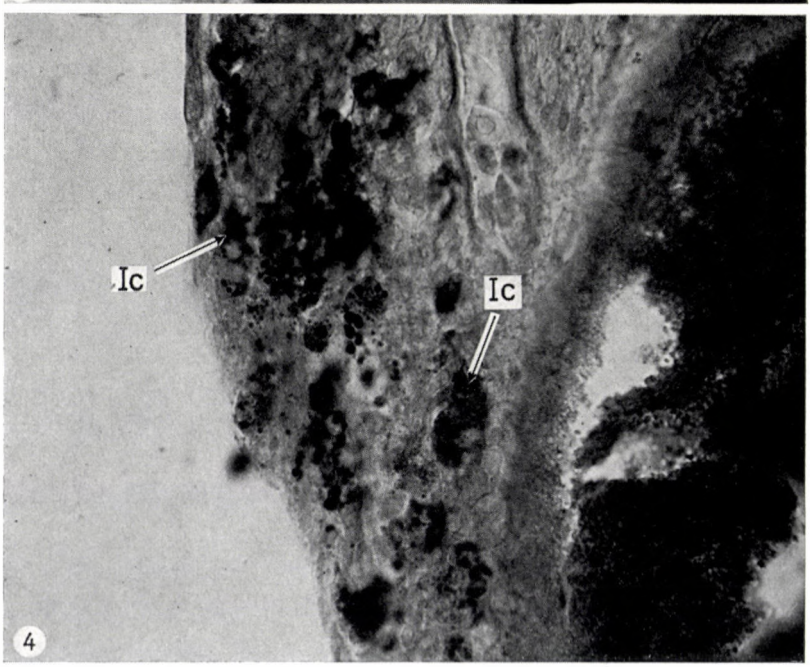


Fig. 3. Large previtellogenic follicles in atresia, showing theca (T), regressing granulosa cells (GC) and epithelial cells (EC) in the ooplasm (O). Technique as in Fig. 1. ×540

Fig. 4. Groups of interstitial gland cells (IC) filled with sudanophilic lipids in the nodules of ovarian stroma. The lipids are left behind after the complete degeneration of large previtellogenic follicles. Technique as in Fig. 1. ×540

destined to undergo atresia is the disappearance of the cortical granules which is shown by the absence of their PAS-positive material (carbohydrates). Instead, there appear many sudanophobic vacuoles in the peripheral portions of the egg (Fig. 5) previously occupied by the cortical granules. The vacuoles (Fig. 5) react negatively to the histochemical techniques used. Such vacuoles are not present in the normal egg follicle. The distribution, morphology and

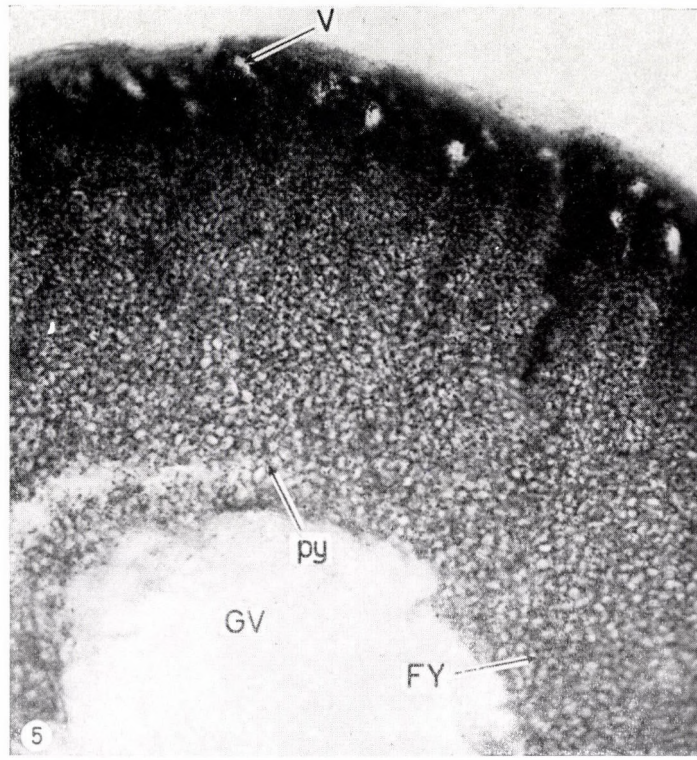


Fig. 5. Higher power view of atretic egg follicle showing the appearance of vacuoles (V) at the periphery of the egg, where yolk elements and pigment granules have started forming abnormal aggregations due to coalescence. The inner portions of the egg contain normal proteid yolk (PY), fatty yolk (FY), and germinal vesicle (GV). Technique as in Fig. 1. ×300

histochemistry of proteid yolk platelets, fatty yolk globules and pigment granules do not differ from those in the normal egg. However, there are seen some signs of coalescence of fatty yolk bodies at the periphery of the egg. Similarly, the pigment granules also seem to form some abnormal aggregations. The egg envelopes are still intact during stage 1 (Fig. 5).

Stage 2. With the advancement of atresia, dramatic changes occur in the atretic follicles which have shrunken by this stage (Fig. 6). The shrinkage is accompanied by further changes in the deutoplasmic inclusions, which form ab-

normal aggregations (Fig. 6). The fatty yolk especially forms a continuous, sudanophilic mass of triglycerides, owing to the coalescence of its various elements which are present as separate bodies in the normal egg and stage 1 of atresia (Fig. 5). The sudanophobic vacuoles of various sizes have now spread throughout the yolky contents of the egg (Fig. 6). Most of the proteid platelets have been broken up and digested (Figs 7, 8), without undergoing any histochemi-

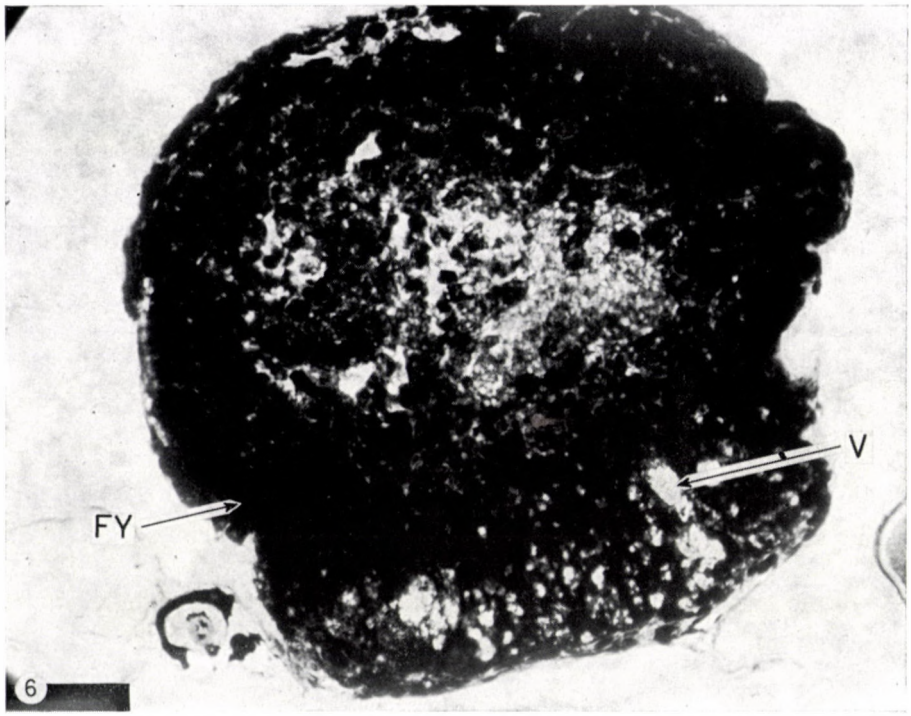
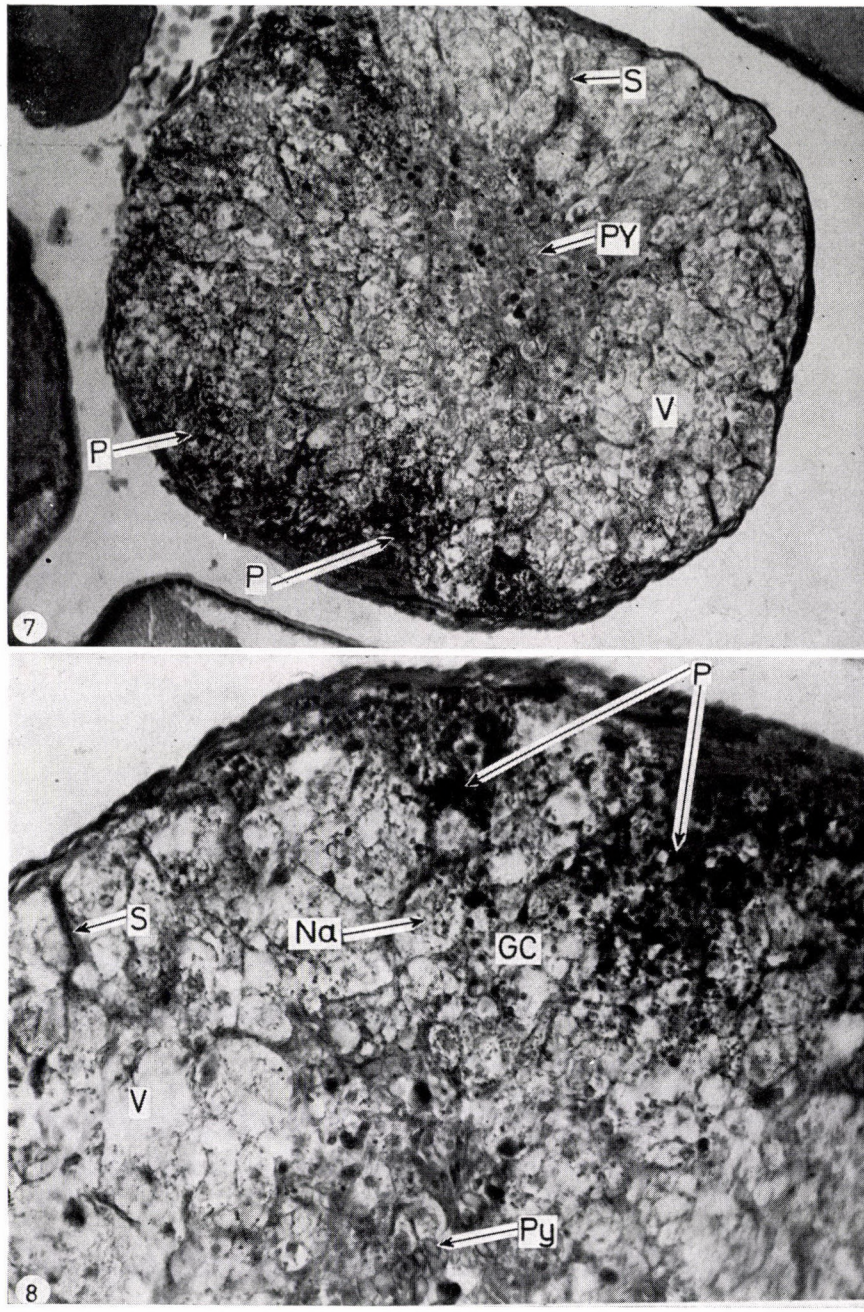


Fig. 6. Higher power view of atretic egg follicle showing large vacuoles (V) throughout the egg. Fatty yolk elements (FY) have coalesced with one another to form continuous sudanophilic masses of variable size. Technique as in Fig. 1. ×150

cal change. During their disintegration, the surface layer consisting of lipoproteins first breaks, leaving the main body (composed of proteins) free to be broken up into small pieces (Figs 7, 8). At this stage of atresia, some of the proteid platelets are still intact in the central portions of the egg (Figs 7, 8). The pigment granules have aggregated into large masses which mostly lie in the outer portions of animal pole of the egg (Figs 7, 8).

During the second stage of atresia, the egg envelopes undergo very conspicuous changes. The regular zona pellucida and follicular epithelium are not seen (Figs 7, 8). The cells of the latter have moved inside the yolky contents of the egg, where they form conspicuous epithelial cells having clear vacuolated



Figs 7—8. Atretic egg follicles in stage 2 of atresia, showing the fragmentation and digestion of proteid yolk by the granulosa cells (GC) having vesicular nuclei (Na) and froathy cytoplasm, abnormal aggregations of pigment granules (P), vacuoles (V) and ingrowths of stroma (S). Most of the proteid yolk bodies have been fragmented and digested, leaving a few ones intact (PY) in the centre of the egg. Formaldehyde-calcium, postchromed in dichromate-calcium, embedded in paraffin, Sudan black B. Fig. 7. ×150; Fig. 8. ×300

cytoplasm and vesicular nucleus (Figs 7, 8). These cells of granulosa origin cannot be distinguished in the frozen gelatine sections of material fixed in 10% formalin or formaldehyde calcium and coloured in Sudan black B (Fig. 6) and red Sudan dyes. After such treatments, the continuous mass of lipids, which is intensely sudanophilic, obscures the epithelial cells. The latter become visible

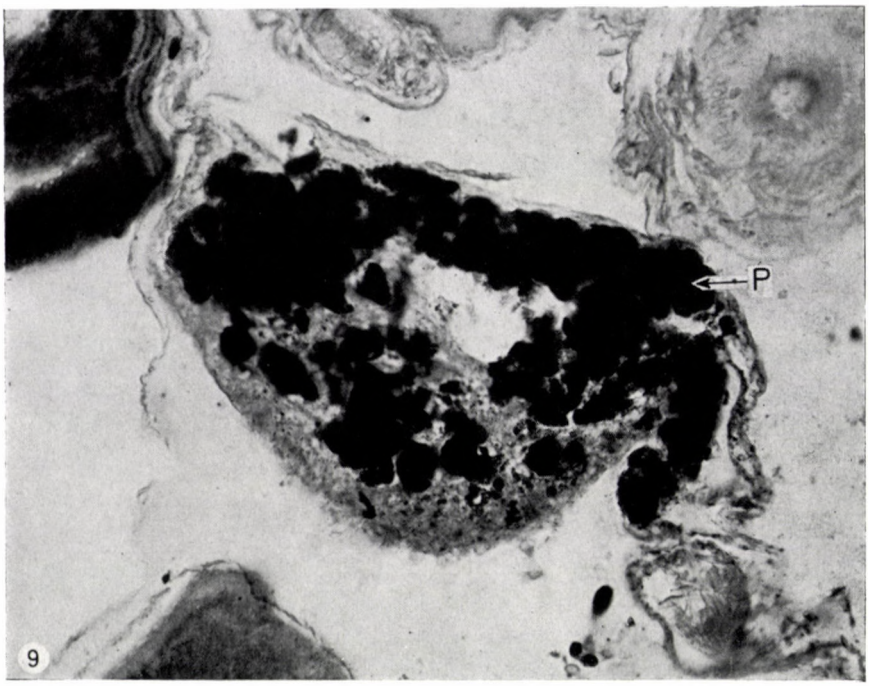


Fig. 9. Aggregations of pigment granules (P) left behind in the ovarian stroma after the complete resorption of yolk elements and granulosa cells in the mature follicles. Technique as in Fig. 1. ×540

after paraffin embedding, owing to the removal of lipids in the ethanol used for dehydration after fixation in Zenker, Carnoy, Bouin's fluid and formaldehyde calcium (Figs 7, 8). The appearance of vacuoles in the cytoplasm of epithelial cells after such techniques suggests that some lipids, which were actually present in their cytoplasm, have been dissolved. Their cytoplasm also contains small fragments of proteid yolk platelets. The various elements of connective tissue, most probably derived from the theca, have permeated throughout the egg contents. The egg at this stage of atresia is surrounded by a thick sheath of connective tissue elements (Figs 7, 8).

Stage 3. By this stage of atresia, all the yolk elements have been digested and removed, leaving behind a mass of pigment material surrounded by the connective tissue elements (Fig. 9). Numerous such masses of various sizes can

be seen in the ovary. The variable sizes of pigment masses suggest that it takes a long time for the disposal of pigment material.

Interstitial gland tissue

The interstitial gland tissue is not much developed in the toad ovary. It occurs in the form of some hypertrophied cells. Connective tissue divides the interstitial gland cells into patches of variable size which are loaded with sudanophilic lipid droplets consisting of either phospholipids or phospholipids and triglycerides. However, during winter, the lipid droplets of cells of thecal origin also give a positive reaction for cholesterol or its esters.

In the amphibian ovary, the atresia of large previtellogenic follicles is closely related to the formation of interstitial gland cells which originate by the hypertrophy of thecal elements as already described. The granulosa cells of these follicles store lipid droplets but ultimately disappear without contributing to the interstitial gland cells.

Discussion

This histochemical study shows that the atresia affects the previtellogenic and vitellogenic follicles of all sizes in the ovaries of *Bufo stomaticus*. With the start of atresia, the structural organization of various components of the follicle is lost. In other words, the different components are all there, but the specific structural order on which the functional capacity of the whole follicle depends is lacking. Atretic follicles of different sizes behave differently.

During atresia (degeneration) of previtellogenic follicles, the peripheral lipid bodies and vacuoles, which have been considered to constitute the nutrient transfer design in the normal growing follicle [8], are not seen. Similarly they disappear in the atretic oocytes of reptilies [9]. The normal mitochondria consisting of lipoproteins are not seen in the ooplasm of degenerating oocytes. However, in the atretic oocytes of reptilies (Squamata), numerous lipid granules consisting of phospholipids and triglycerides, are simultaneously formed [9]. The lipids of similar histochemical nature were only formed in the very young oocytes of the present material, which also developed some abnormal aggregations of yolk nucleus substance consisting of protein, lipoprotein and RNA. The atretic oocytes of snakes show larger aggregations of yolk nucleus substance [9].

The follicular epithelial cells of atretic previtellogenic follicles store some lipid droplets, consisting of phospholipids and triglycerides. Similar lipids of atresia show larger accumulations in the granulosa of atretic follicles in reptiles [9] and mammals [20a, 20b, 20c]. The lipid droplets in the atretic granulosa of the mammalian ovary also store cholesterol or its esters, in addition to phospholipids and triglycerides.

The ooplasm, germinal vesicle and granulosa cells of small follicles are gradually reabsorbed without showing conspicuous histochemical changes. However, the granulosa cells of large previtellogenic follicles seem to play an important role in the digestion and removal of ooplasmic contents. This is in agreement with the histochemical studies on the follicular atresia in the reptilian ovary [9].

With the resorption of oocyte and granulosa cells in the large previtellogenic follicles, there are some cells formed by the hypertrophy of thecal elements. These hypertrophied cells develop lipid droplets consisting of either phospholipids or phospholipids and triglycerides, or phospholipids, triglycerides, and cholesterol or its esters. The cells of comparable origin are also formed in the ovaries of reptiles and mammals [9—17, 20a, 20b, 20c] in which they vary greatly in their amount, distribution and permanency. However, they store sudanophilic lipids consisting of either phospholipids or phospholipids and triglycerides or phospholipids, triglycerides and cholesterol or its esters. These hypertrophied cells of thecal origin have been designated as the interstitial gland cells in the ovaries of reptiles and mammals [9—17, 20a, 20b, 20c]. The current histochemical study shows that the interstitial gland cells of thecal origin are also formed in the amphibian ovary.

It is generally believed that when cholesterol or its esters are accumulated abundantly in steroid-producing cells, hormone storage is taking place, and when the amount decreases hormone is released [10, 12, 14, 20a]. It can now be added that during winter the interstitial gland cells of *Bufo stomaticus* begin to store hormone precursor (cholesterol or its esters) rather than secrete hormone and the hormone secretion occurs in the interstitial gland cells of summer ovaries, which do not show storage of cholesterol or its esters. These lipid changes in the interstitial gland cells also suggest that, in comparison to winter, there are high gonadotrophin levels during summer, and these are responsible for the depletion of cholesterol or its esters. The high gonadotrophin levels are now well known to cause the mobilization of hormone precursor [10, 12, 13, 14, 20a, 20b, 20c].

The first change, which occurs during the atresia of mature follicles filled with yolk, is the disappearance of cortical granules. This indicates a greater sensitivity of the latter towards atresia. The sudanophobic vacuoles, which appear in place of cortical granules, seem to be derived from the latter. In the previous literature there are some suggestions that the cortical granules are some kind of lysosomes [24]. This suggestion seems to be supported by the present study as the sudanophobic vacuoles originated from the cortical granules first initiate the fragmentation and dissolution of yolk elements of peripheral ooplasm. During atresia the fatty yolk elements become watery and coalesce with one another to form a continuous sudanophilic mass. This suggests that some physico-chemical changes occur in the organization of yolk elements dur-

ing atresia. Similar changes occur in the yolk elements of reptilian eggs undergoing atresia [9]. The yolky contents of mature follicles are gradually digested and removed by the granulosa cells which have been found to contain small fragments of both the lipid and proteid yolk elements in their cytoplasm. Phagocytic activity of the granulosa cells of the ovary has also been reported to exist in a wide variety of vertebrates which have been studied with the routine histological techniques [3, 9].

The degenerating mature follicles in the amphibian ovary have been usually designated as the corpora atretica or "preovulatory" corpora lutea which are believed to secrete steroid hormones [26]. The current study shows that they do not perform any endocrine function as the hormone precursor (cholesterol or its esters) has not been observed in them at any stage of their development and degeneration. This is strongly supported by the fact that the hormone precursor stores abundantly in the regressing corpora lutea of mammals [7, 18, 19, 20, 20c], which are well-known to secrete steroid hormones. The phagocytic granulosa cells of degenerating mature follicles in the amphibian ovary are mainly concerned with the digestion and disposal of yolk elements as already stated. These mature follicles apparently become atretic due to the low levels of gonadotrophins during winter when they seem to undergo degeneration in large numbers. They are not formed in response to a gonadotrophic stimulus from the pituitary gland [26].

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HISTOCHEMISCHE UNTERSUCHUNG DER FOLLIKELATRESIE AM AMPHIBIENOVAR

Die Follikelatresie und die Probleme der interstitiellen Drüsenzellen im Krötenovar *(Bufo stomaticus)* wurden mit histochemischen Methoden untersucht. Die Atresie läßt sich an Follikeln jeder Größe beobachten, jedoch im Winter ist die Atresie der reifen Follikel eine besonders häufige Erscheinung. Einzelheiten der morphologischen und histochemischen Veränderungen am Ooplasma und an den Eihäuten werden beschrieben. Bei der Atresie der großen previtellogenischen Follikeln sind manche aus der Theca stammende Zellen hypertrophiert und mit Lipidtröpfchen angefüllt, die anfangs aus Phospholipiden, dann aber aus Phospholipiden und Triglyceriden bestehen. Sie bilden die interstitiellen Drüsenzellen, die im Winter außer anderen Lipiden auch Cholesterol oder dessen Esther anhäufen. Die Oozyten, die zona pellucida und die Granulosazellen regredieren und verschwinden schließlich. Den letzteren kommt in der Verdauung der Dotterelemente und deren Entfernung aus den degenerierenden reifen Follikeln eine wichtige Rolle zu; letztere weisen keinerlei gemeinsame cytochemische Eigenschaften mit dem Corpus luteum von Säugern auf. Der Histochimismus der atretischen Follikeln von Kröten wurde mit dem Histochimismus der Follikeln von Reptilien und Säugern verglichen und gegenübergestellt. Die interstitiellen Drüsenzellen des Amphibienovars werden im Lichte der jüngsten histochemischen Untersuchungen besprochen, die an analogen Zellen der Reptilien- und Säugerovarien ausgeführt wurden.

ГИСТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ АТРЕЗИИ ФОЛЛИКУЛОВ В ЯИЧНИКАХ ЗЕМНОВОДНЫХ

Атрезия фолликулов и проблемы интерстициальных клеток желез в яичниках жабы *Bufo stomaticus* были изучены гистохимическими методами исследования. Атрезия поражает фолликулы всех размеров, однако, в зимний период атрезия зрелых фолликулов наблюдается очень часто. Дается описание подробностей морфологических и гисто-

химических изменений ооплазмы и оболочек яиц. Во время атрезии крупных превителлогенических фолликулов, некоторые клетки, происходящие из оболочки фолликула, гипертрофированы и наполнены липидными капельками, состоящими в начале из фосфолипидов, а затем из фосфолипидов и триглицеридов. Они составляют интерстициальные клетки желез, накопляющие в зимний период, наряду с другими липидами, также холестерол или его эфиры. Ооциты, прозрачная зона и гранулезные клетки окончательно регрессируют и исчезают. Последние играют важную роль в переваривании и удалении желточных элементов из дегенерирующих зрелых фолликулов, не имеющих никаких общих цитохимических черт с желтым телом млекопитающих. Гистохимизм атретических фолликулов жабы была сопоставлена и сравнена с гистохимизмом атретических фолликулов пресмыкающихся и млекопитающих. Интерстициальные клетки желез яичников земноводных рассматриваются в свете новейших цитохимических исследований, проводившихся на аналогичных клетках яичников пресмыкающихся и млекопитающих.

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ON THE MECHANISM OF MAST CELL FORMATION

EFFECT OF GLUCOCORTICOIDS ON THE MAST CELLS OF NORMAL AND THYMECTOMIZED RATS

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Synopsis

Following thymectomy, young mast cells decrease in number in the peritoneal fluid of rats, indicating that the thymus may be involved in the genesis of mast cells [2]. On prolonged glucocorticoid treatment the peritoneal mast cells are destroyed while, at the same time, in the thymus and the lymph nodes the young mast cells increase in number. Cells with serotonin fluorescence show a simultaneous increase in number in the thymus, lymph nodes and the peritoneal fluid. The mechanism of mast-cell formation and the role played in this process by the thymus and the glucocorticoids are discussed. The present work includes histochemical, autoradiographic and fluorescence- and electron-microscopic investigations. The results were evaluated statistically.

Introduction

The origin of mast cells was investigated in earlier experiments [7—10, 14]. It has been found that mast cells are produced in the thymus and in lymph nodes. The formation of mast cells in these organs was preceded by an accumulation of PAS-positive substances that were taken up by large or medium-sized thymocytes and reticular cells, which changed into mast cells in the course of this process [8]. Although such phenomenon cannot be observed in lymph nodes under physiological conditions, a great amount of mast cells was found in the follicles after treatment with certain hormones [12], this means that lymph nodes not only store but also produce mast cells.

Hormones may both increase and decrease the number of mast cells, depending not merely on the hormone employed but on the site and the method of investigation as well [26], a circumstance that is presumably responsible for existing contradictions concerning the effect of certain hormones on mast cells [26]. This applies to corticosteroids, too. We found in earlier studies that glucocorticoids promoted mast-cell formation in the thymus [12, 14], while — peripherally (in the subcutaneous connective tissue) — initial increase in the number of mast cells was followed by a decrease owing to degranulation. The effect of mineralocorticoids appeared to be opposite but less marked [11].

In order to be able to follow the stages of mast-cell formation and degranulation and to judge the effect of a given factor on these processes a method is necessary allowing a quantitative (mathematical) evaluation of the observed phenomena, further a histochemical procedure which makes a differentiation of the various mast-cell types, i.e., the different phases of their maturation, possible. Recent investigations have shown that such methods and procedures may be evolved. The present experiments were designed to study the role of the thymus and the glucocorticoids as well as the combined effect of these factors in the regulation of mast cells.

We wanted to elucidate the following problems.

(1) Influence of the thymus — supposedly the primary producer of mast cells — on the formation and fate of peritoneal mast cells.

(2) Effect of glucocorticoids on the formation and disintegration of mast cells in the peripheral and especially in the lymphatic organs.

(3) Influence of the presence or absence of thymus on the effect of glucocorticoids on mast cells.

When starting the experiments we thought that simple histochemical analyses combined with the registration of mast-cell counts would suffice for the solution of these problems. Their intricacy made it, however, necessary to resort to fluorescent- and electron-microscopic, further to qualitative and quantitative isotope methods as well. Although these combined methods still have left some problems unsolved, they yielded at least information on fundamental principles.

Material and method

Separate and combined effect of thymectomy and glucocorticoids

Forty adult Wistar R rats of about 200 g body weight were divided into 6 groups:
control,
thymectomized,
prednisolone-treated,
cortisone-treated,
thymectomized + cortisone-treated,
thymectomized + prednisolone-treated.

Thymectomy. The skin of the neck was incised, the salivary glands were retracted, and an incision of about 8 cm was made in the midline of the sternum. After drawing apart the two ends of the manubrium, we removed the thymus together with its capsule. The success of the operation was checked with the naked eye or histologically at the sacrifice of the animals. Treatment was started 46 days after the operation.

Prednisolone (di-Adreson-F-aquosum-Organon) was administered intravenously in doses of 5 mg/100 g body weight. Sample was taken 60 min. after the injection.

Cortisone (Adreson-Organon) was administered intramuscularly for three days in daily doses of 5 mg/100 g body weight. No injection was given on the fourth day, and sample was taken on the fifth day.

Before taking sample we injected into the abdominal cavity 10 ml of a fluid of the following composition: sodium chloride, 0.7 g; sodium citrate, 1.5 g; distilled water, 100 ml. The fluid was kept in the abdominal cavity for three minutes during which the animals were narcotized with ether. The abdominal cavity was then opened and the fluid was sucked off. One drop of each suspension was transferred to a fat-free slide, and the preparations were

immediately placed in a 37 °C thermostat. It should be noted that cells are damaged if drying is either too quick (at 45 to 50 °C) or too slow (at room temperature).

Dried preparations have to be fixed immediately since even a delay of 1 or 2 days affects the results. Carnoy's fluid served as fixative. Fixed material should be stained as soon as possible; if staining is delayed, preparations have to be stored in a dry condition: they must not be kept in water and not even left in a moist condition. We employed SPICER's [32] alcian blue-safranin staining method as modified by BALOGH [2]. Essentially, the modification consists in the addition of iron alum to the stain. It stabilizes the stain, reduces the risk of overstaining and makes the method more specific. The pH of the dye solution was 1.02.

A total of 2,000 to 3,200 cells were counted per group. The principles of cell counting are discussed in detail below.

Thymus and lymph node samples were fixed in Carnoy, embedded in paraffin, and the sections were stained with alcian blue-safranin as described above.

Demonstration of the appearance of cells containing serotonin

Sixteen adult male rats divided in two groups, were used for this series of experiment. Four animals served as controls, while the 12 members of the second group received for 3, 6 or 8 days cortisone in doses of 5 mg/100 g body weight. As the treatment has finished, we washed the abdominal cavity of the control and test animals as described in the foregoing. Thick drops were transferred to slides or, else, the lymph nodes and the thymus were taken out, immediately frozen in carbon-dioxide snow and sectioned in the cryostat. Sections were dried on silica gel; both the sections and the thick drops were then fixed in 80 °C paraformaldehyde vapour for one or 24 hrs according to the method of FALCK [18, 19].

Preparations were examined under the fluorescent microscope.

Uptake of $Na_2{}^{35}SO_4$ by peritoneal mast cells

Twenty adult male rats were used. Members of the first group of this series received cortisone (in doses as described above) for three days, those of the second group served as controls. On the fourth day, members of both groups were intraperitoneally injected with $Na_2{}^{35}SO_4$ dissolved in saline (200 μCi/kg). The working solution contained 1 mCi/ml. On the fifth day, the abdominal cavity of the animals was washed as described above and the specific activity of both the whole suspension and the supernatant fluid determined. Specimens for the measurement of radioactivity were prepared as follows. Five ml of washing fluid was withdrawn from each animal, 1.0 ml thereof to be used as whole suspension and the rest to be centrifuged at 800 to 1,000 r.p.m. One ml of the supernatant fluid was separated following centrifugation whereafter both the samples of whole suspension and those of the supernate were identically prepared for scintillation measurements. We added to each sample 0.4 ml of the molar methylalcoholic solution of the detergent Hyamine-OH 10×. As soon as the suspension became clear, 10 ml of a scintillator fluid (POPOP + PPO + naphtalene + dioxane) was added, and radioactivity measured in a Packard TRICARB instrument. Other samples were stained with toluidine blue, and the mast cells were counted in Bürker's chamber.

After centrifuging part of the washing fluid at 800 r.p.m., the supernate was discarded and the sediment was resuspended in Tyrode's solution by mild shaking. It was repeatedly centrifuged and washed in Tyrode's solution until the disappearance of radioactivity of washing fluid.

We prepared thick drops from the washed suspension, fixed them in absolute methanol, stained them with alcian blue-safranin and covered with Kodak AR 10 stripping film. Exposure lasted 4 to 6 weeks when development was performed in Kodak D 19b and fixation in the Kodak acid fixative; after being washed in tap water, the material was mounted in the usual way.

Electron-microscopic examination of peritoneal mast cells

The abdominal washing fluid of adult male rats was transferred to a sma ll conica plastic tube and centrifuged at 500 r.p.m. Some animals were untreated, others had been pretreated with cortisone for three days. After centrifugation the material was resuspended in Parker T. C. 199, re-centrifuged and fixed in a 4 : 1 mixture of 2% formalin and 25% glutaraldehyde [33]. A solution of 0.07 M sodium cacodylate (pH = 7.1) served as buffer. Fixation lasted two hours at room temperature, washing (likewise with sodium cacodylate as above) 30 min. Refixation was performed in a refrigerator with 1% osmium tetroxide for 20 min. It was followed by dehydration by ethyl alcohol, treatment with propylene dioxide

and embedding in araldite. Thin sections were made by means of an ultramicrotome, type Reichert OMU2. After counterstaining with uranyl acetate and lead citrate, the preparations were examined in an electron microscope, type JEM 6C.

The material, repeatedly centrifuged, was kept in the plastic tube all along, i.e., until embedding, when the plastic envelope was removed, leaving the block free.

Results

Cytological examination of mast cells in the peritoneum and in the lymphoid organs of thymectomized, glucocorticoid-treated and thymectomized and glucocorticoid-treated animals

Certain cells in the thick drops obtained from the peritoneal fluid and stained with alcian blue-safranin showed specific staining. We distinguished five types of these cells, four of which were identical with the mast cells classified by COMBS and co-workers [5].

The narrow cytoplasm of cells stainable only with alcian blue was hardly granular. These cells resembled medium-sized lymphocytes (Fig. 1). The cells containing also safranin-positive granules were larger, while the largest cells stained with safranin only. Some medium-sized cells contained both alcian blue- and safranin-positive granules; one type of granules was predominant in certain, the other type in other, cells. Damaged mast cells, constituting the fifth type not included in the classification of COMBS and co-workers (Fig. 2), were observed almost exclusively in test animals and practically never in controls.

Histological preparations made from the thymus of the latter showed in the connective tissue chiefly mast cells with red granules, while a small number of mast cells with alcian-blue-positive granules were observed in the subcapsular area. Both types, especially the subcapsular blue cells, increased considerably in number after three days of cortisone treatment (Fig. 3). The same phenomenon was found in the lymph nodes (Fig. 4) where the majority of mast cells were situated in the follicles. Treatment with cortisone increased the number of mast cells in the lymph nodes even in the thymectomized animals. Prednisolone produced no appreciable change either in the thymus or the lymph nodes.

Mathematical-statistical analysis of peritoneal mast-cell values obtained in thymectomized glucocorticoid-treated and thymectomized and glucocorticoid-treated animals

With a slight modification, we distinguished the various categories of mast cells according to the classification of COMBS and co-workers [5]. Group 1 contained nongranular or hardly granular small lymphoid cells which stained exclusively blue with alcian blue-safranin. Group 2 consisted of cells containing

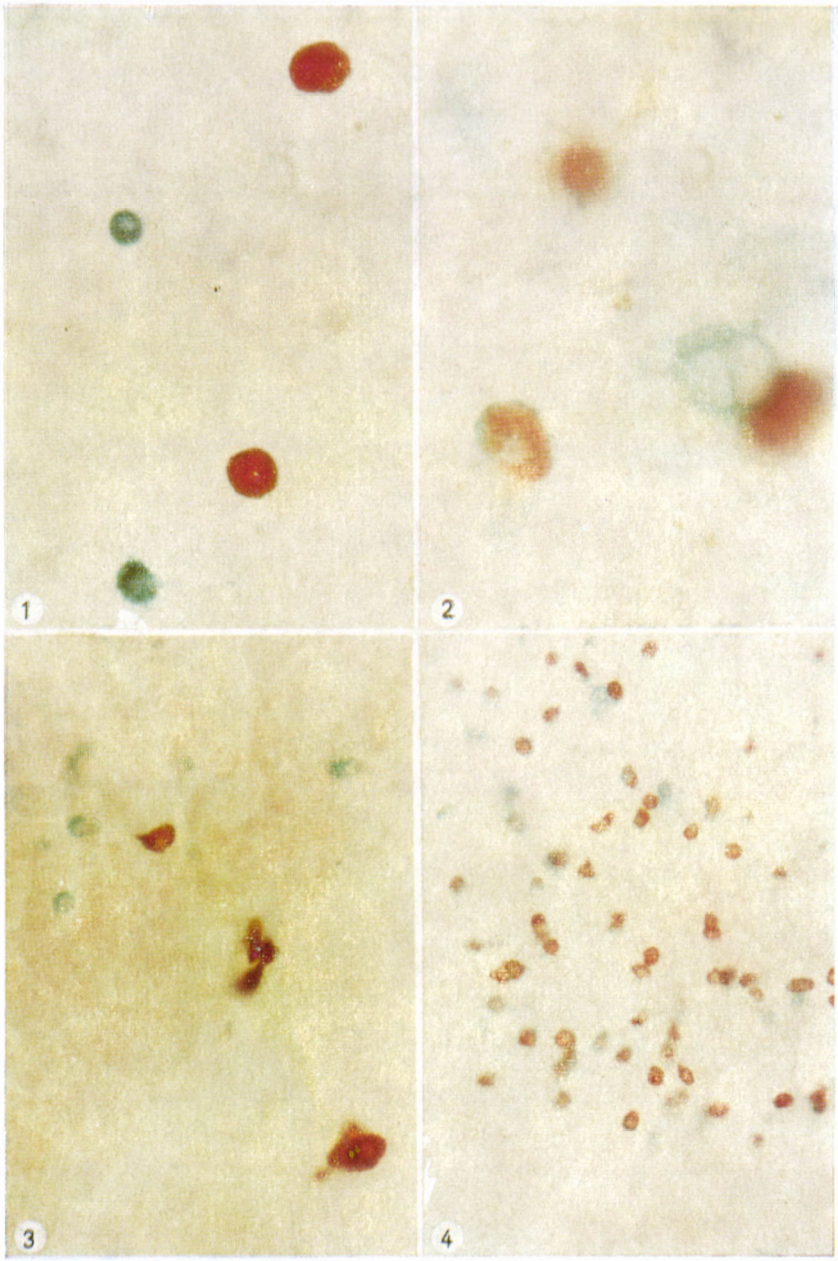


Fig. 1. Left, below: medium-sized blue mast cells suggestive of lymphocytes. Right, below: larger mast cells showing predominance of blue granules. Above: mature red mast cells. Alcian blue-safranin. ×450

Fig. 2. Mature mast cells containing only red granules and seriously damaged blue mast cell. Alcian blue-safranin. ×1000

Fig. 3. Section of thymus removed from rat after 3-day cortisone treatment. The connective tissue contains red, the subcapsular zone chiefly blue, mast cells. Alcian blue-safranin. ×450

Fig. 4. Cortex of lymph node after a three-day treatment with cortisone. Note large number of blue and red mast cells. Alcian blue-safranin. ×160

both blue- and red-staining granules irrespective of which colour was predominant. Group 3 consisted of mature mast-cells containing practically only red granules. To group 4 belonged disintegrating cells which stained blue and were large; their cell membrane was impaired or, else, the cells exhibited some other damage (enlargement of intergranular space, considerable increase in size, peripheral localization of blue staining). These four groups of cells were taken into consideration in the mathematical-statistical analysis.

The nature of our data did not enable us to postulate some known type of distribution and compare statistical characteristics of our observations relating them to parameters thereof. We cannot for instance sufficiently describe by any kind of average the location of the values obtained for the particular groups.

Comparisons of cell-type frequencies between groups are not quite independent as regards either the four cell types or the six experimental possibilities (control + five experimental groups). Nevertheless, the general trends can be recognized by means of all the results regarded as a whole.

Table 1

Results of Wilcoxon's test

Comparison of treatments					
I	II	1	2	3	4
Thymectomy	No treatment	−−	∅	∅	+
Prednisolone	No treatment	−−	++	−	∅
Cortisone	No treatment	−−	−−	−−	++
Cortisone	Prednisolone	−−	−−	∅	++
Thymectomy + prednisolone	No treatment	−−	−−	−	++
Thymectomy + cortisone	No treatment	−−	−−	∅	++
Thymectomy + cortisone	Thymectomy + prednisolone	−−	∅	∅	∅
Thymectomy + prednisolone	Thymectomy	∅	−−	−	++
Thymectomy + cortisone	Thymectomy	∅	−−	−	++
Thymectomy + prednisolone	Prednisolone	−−	−−	∅	++
Thymectomy + cortisone	Cortisone	−	∅	∅	∅

Explanation

Treatment in column I resulted in a
++ = highly significantly higher,
+ = significantly higher,
∅ = not significantly different,
− = significantly lower,
−− = highly significantly lower,
proportion of the designated cells than treatment in column II
1, 2, 3 and 4: designation of cells types.

Table 2

Uptake of $Na_2{}^{35}SO_4$ by peritoneal mast cells

	Whole c.p.m./ml	Supernate c.p.m./ml	Significance	Mast cell count/ml
Test animals	$3.31 \cdot 10^3$	$1.61 \cdot 10^3$	$t = 2.582$ $P < 0.02$	47.7
Controls	$3.58 \cdot 10^3$	$3.11 \cdot 10^3$	$t = 1.683$ $P > 0.1$	15.0
Significance	$t = 0.241$ $P > 0.5$	$t = 5.086$ $P < 0.001$		$P < 0.01$ $x^2 = 115.2$

In consideration of the foregoing and also because the total number of cells examined in the individual groups was approximately the same, we compared — according to their relative frequency (percentage) — within each cell type the results of pairs of treatments the confrontation of which was well amenable to interpretation. The method employed in this respect aimed only at the examination of the shifts in one direction with reference to the total value of distributions. This is the Wilcoxon (Mann-Whitney) test: the data in respect of both specimens to be compared are serially arranged in the order of magnitude, and the serial numbers for each specimen are then summed up. The test itself consists in determining whether, if the specimens had been taken from identical distributions, the probability would be high enough ($> 95\%$ or $> 99\%$), of the above two sums being more proportionately distributed between the two specimens than the sums actually obtained. If the table shows a high probability, the deviation (in the given direction) is regarded as significant or highly significant; at the $> 95\%$ and $> 99\%$ levels the hypothesis of identical distributions is rejected in favour of a real deviation. Results are assembled in Table 1.

Demonstration of the appearance of cells containing serotonin

After having been fixed in paraformaldehyde for an hour, the thymus of control animals (Fig. 5) showed a moderate proportion of cells emitting serotonin fluorescence. The nature of fluorescence technique applied to cryostat sections made it difficult to determine the quality of these cells. Most were large and seemed to be situated preferentially in the subcapsular zone; they resembled reticular cells rather than lymphocytes. A three-day treatment with cortisone sufficed to considerably increase their number, and it was then possible to identify both the reticular cells and the large and medium sized thymocytes (Fig. 6). After a cortisone treatment of six days there were practically only serotonin-containing cells in the cortex of the thymus (Fig. 7). The picture

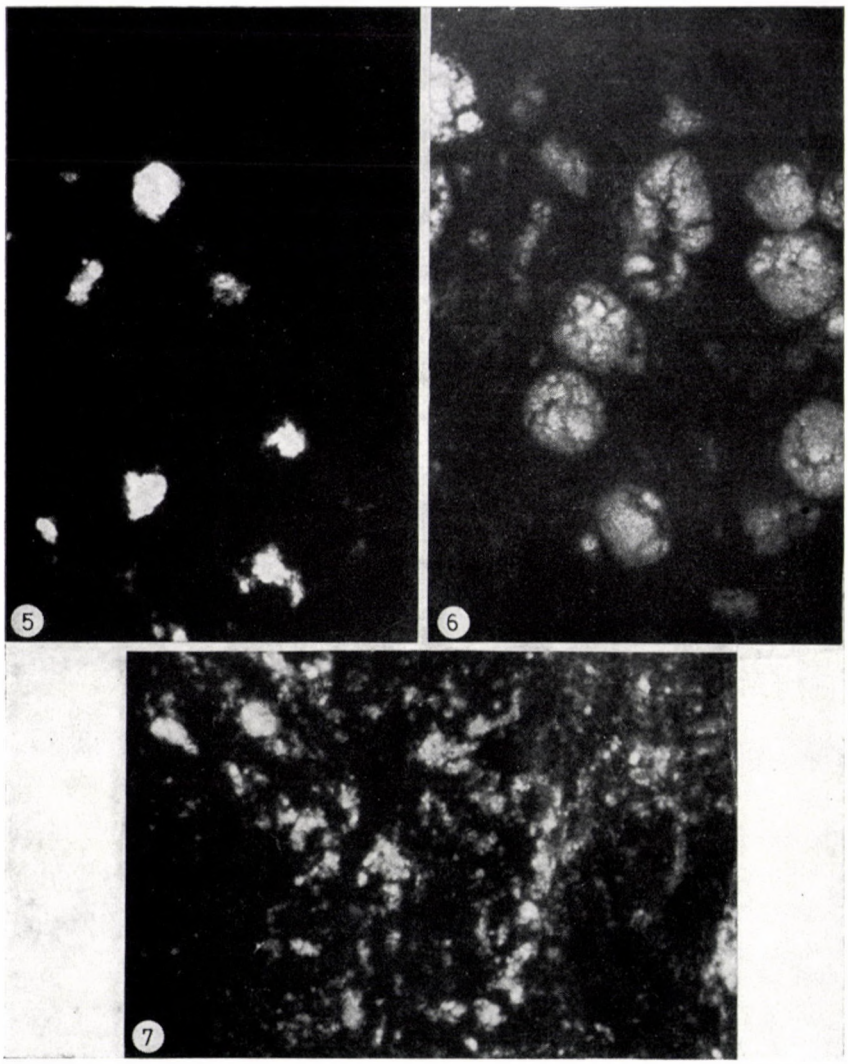


Fig. 5. Fluorescence photomicrograph of a section prepared from control thymus. A few fluorescent cells are visible. One-hour fixation in paraformaldehyde. ×250

Fig. 6. Fluorescence photomicrograph of section prepared from thymus of rat after 3-day treatment with cortisone. There is a large number of fluorescent cells. One-hour fixation in paraformaldehyde. ×250

Fig. 7. Fluorescence photomicrograph of section prepared from thymus of rat after 6-day treatment with cortisone. Practically all cells show fluorescence. One-hour fixation in paraformaldehyde. ×100

remained unchanged as long as to the eighth day of treatment. In the lymph nodes the number of cells was considerably lower.

Only occasional serotonin-positive cells were found in the peritoneal fluid of the control animals (Fig. 8), whereas large numbers thereof were observ-

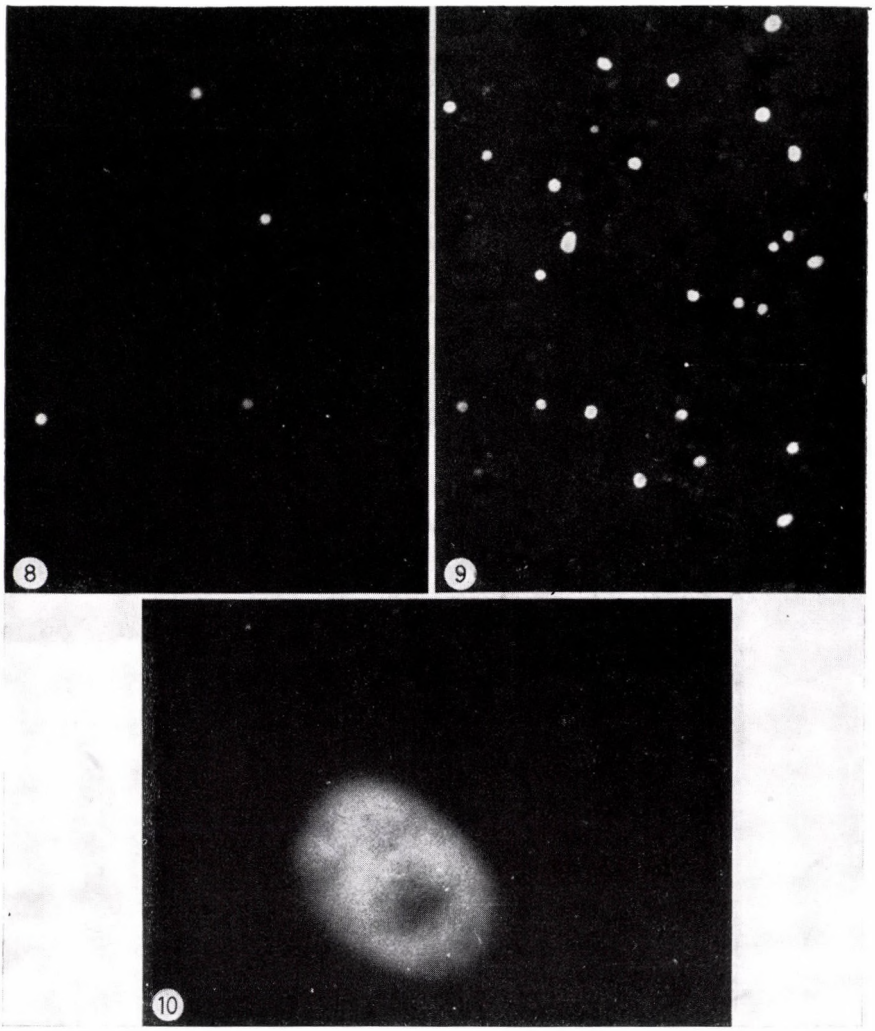


Fig. 8. Fluorescence photomicrograph of peritoneal fluid obtained from control animal. There are hardly any fluorescent cells. One-hour fixation in paraformaldehyde. ×100

Fig. 9. Fluorescence photomicrograph of peritoneal fluid obtained from rat after 3-day treatment with cortisone. Note numerous large and small fluorescent cells. One-hour fixation in paraformaldehyde. ×100

Fig. 10. Fluorescence photomicrograph of peritoneal fluid obtained from rat after 6-day treatment with cortisone. It can be clearly seen that there is no fluorescence over the nuclear area of the cell at the right side. One-hour fixation in paraformaldehyde. ×630

ed after a three-day cortisone treatment (Fig. 9). As seen in Fig. 10, it was only the cytoplasm which contained the fluorescent substance.

By the 24th hour of paraformaldehyde treatment the number of fluorescent cells reached a multiple of that seen after the first hour both in the organs and in the thick drops (Figs 11, 12).

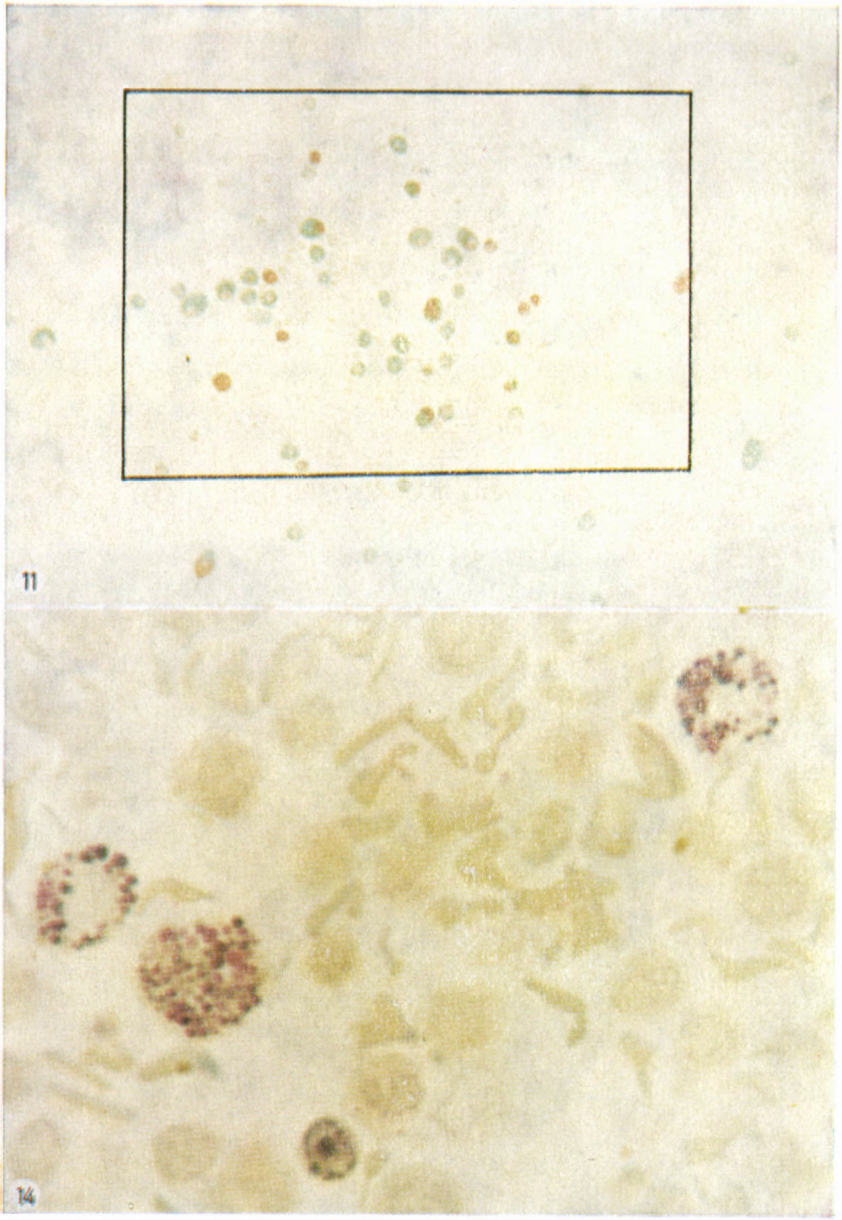
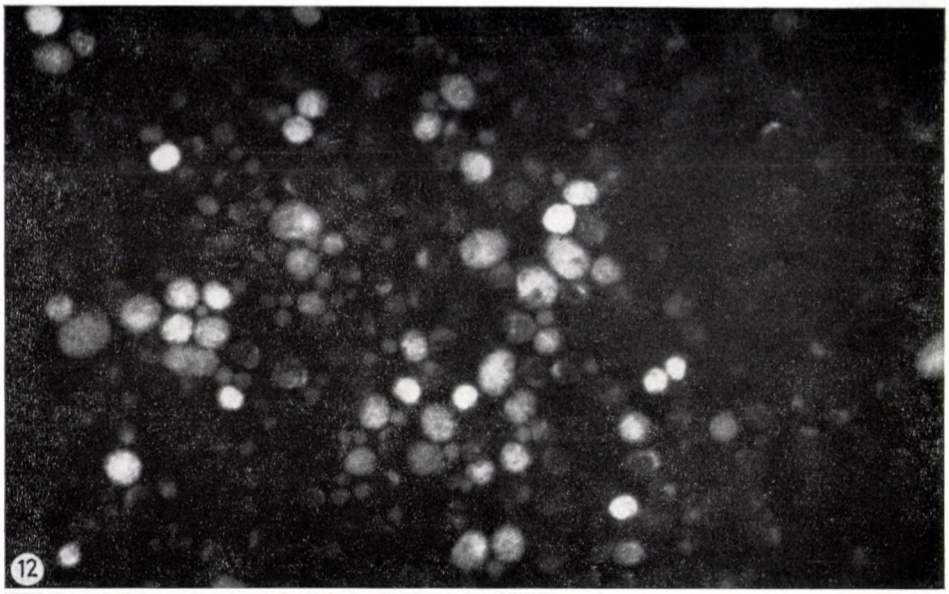


Fig. 11. Peritoneal fluid of rat after 3-day treatment with cortisone. Red and blue mast cells are well-visible. Alcian blue-safranin. Fixation in paraformaldehyde for 24 hours. ×250

Fig. 14. Half-thick preparation of block made from peritoneal mast cells obtained from rat treated with cortisone. Toluidine blue. ×1000



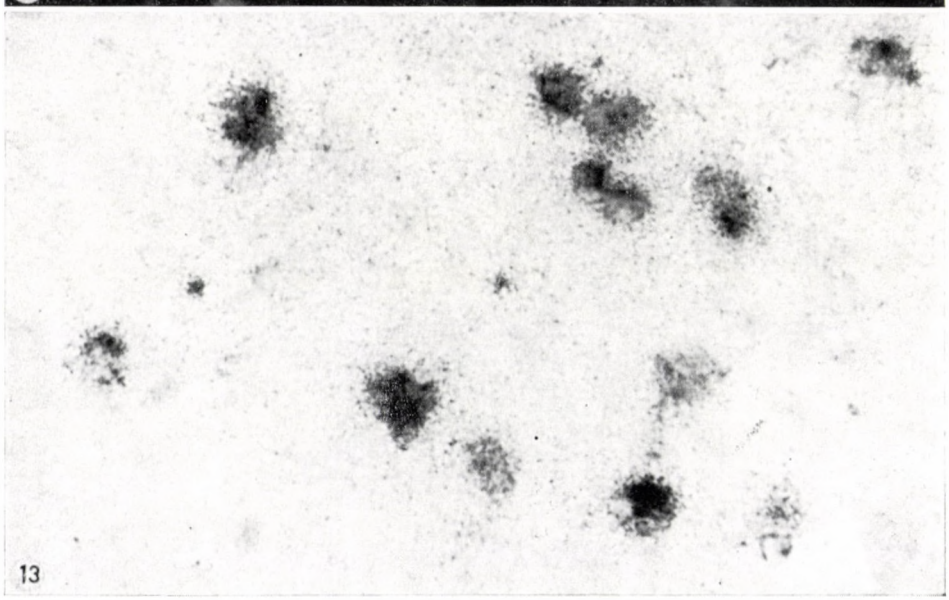


Fig. 12. Fluorescence photomicrograph of inset in Fig. 11 prior to staining. Identical cel's are clearly perceptible. Safranin-positive mast cells show stronger fluorescence. Cells with pale fluorescence did not stain with either alcian blue or safranin, and some strongly fluorescent cells are invisible in the preparation stained with alcian blue-safranin. Fixation in paraformaldehyde for 24 hours. ×250

Fig. 13. Autoradiograph of peritoneal fluid obtained from rat after 3-day cortisone treatment. Note precipitation of silver grains above alcian-blue positive cells. Alcian blue-safranin. ×450

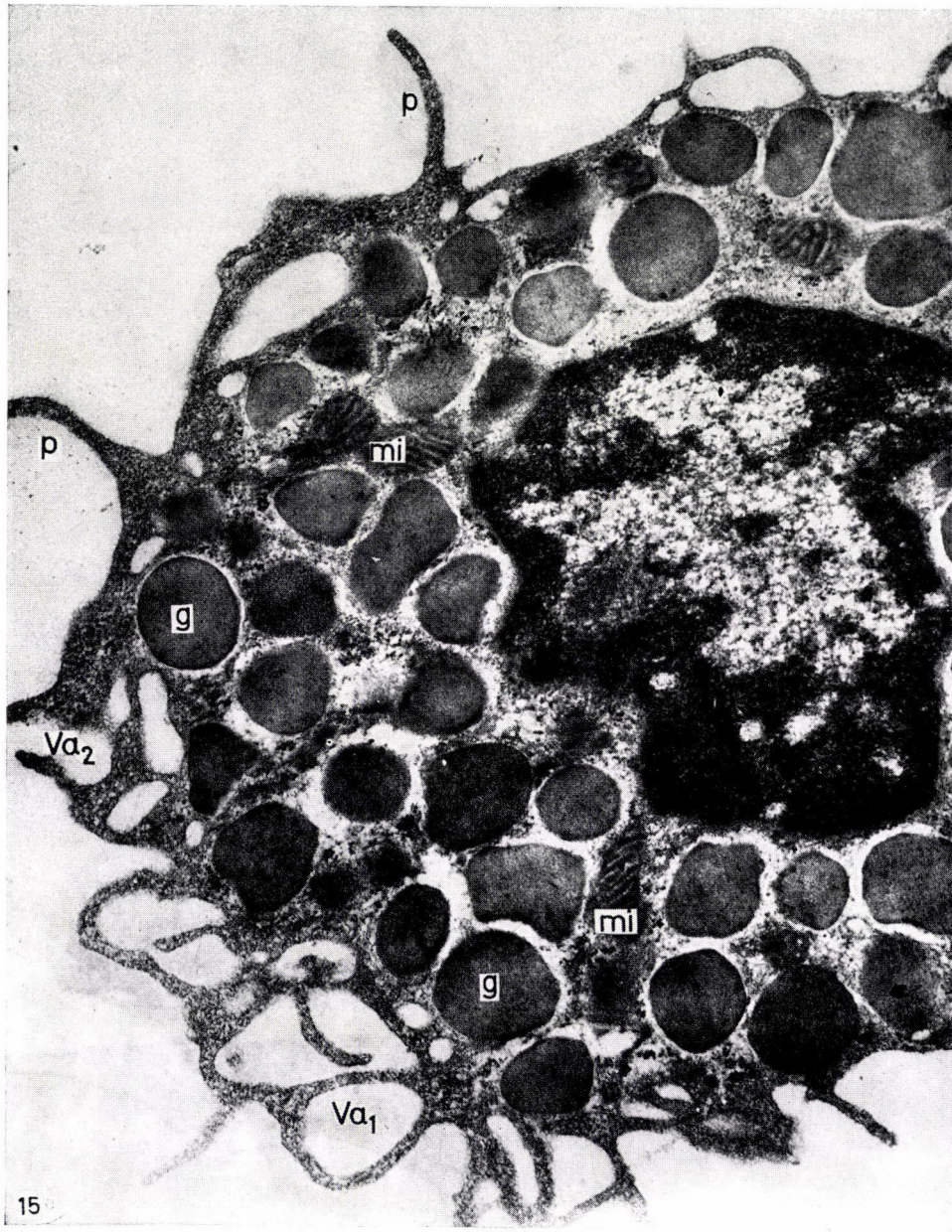


Fig. 15. Electron-micrograph of peritoneal mast cell. Granules are intact, although the cell was obtained from animal after a 3-day treatment with cortisone. g = granule; mi = mitochondrium; p = cytoplasmic process; Va_1 = detached vacuole; Va_2 = empty vacuole. ×25,750

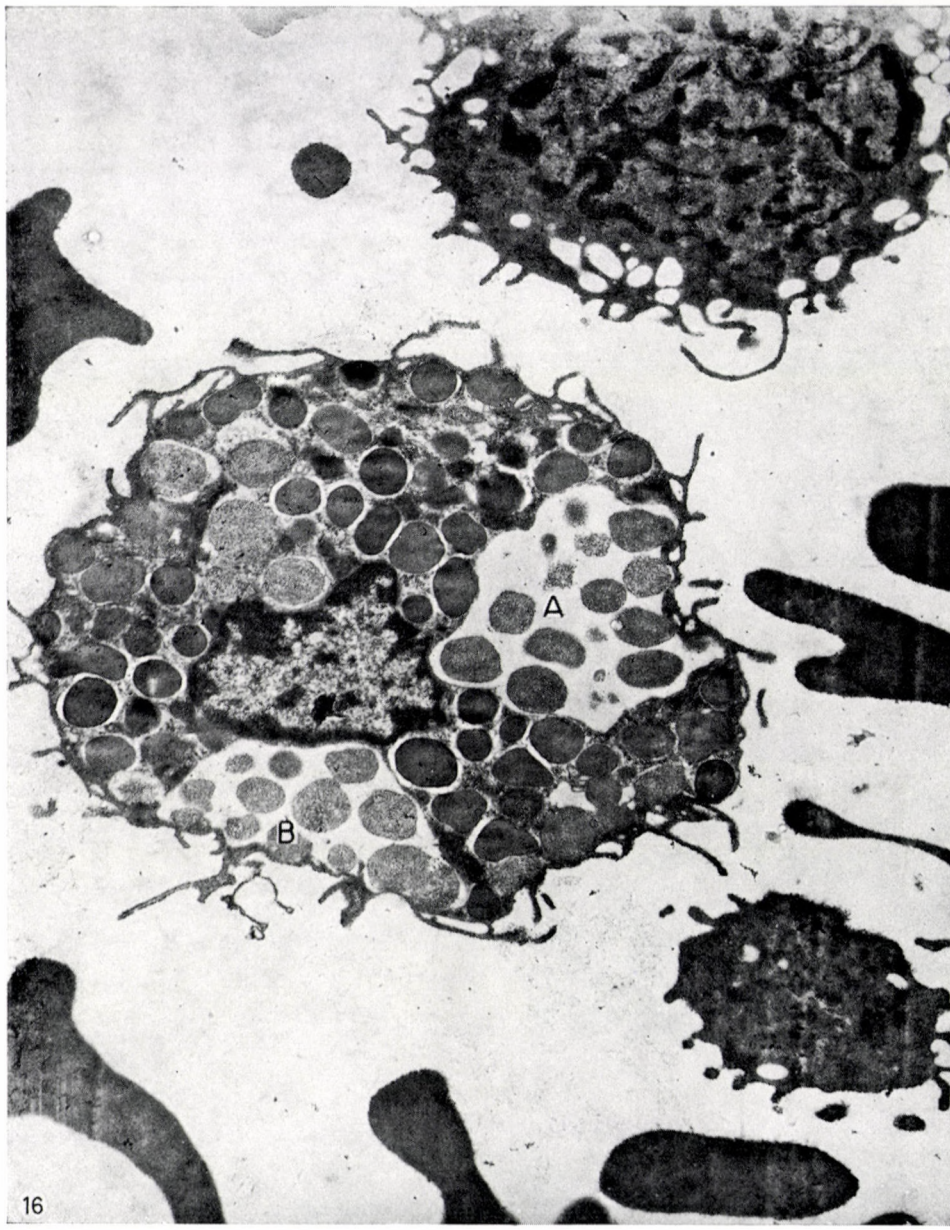


Fig. 16. Electron-micrograph of mast cell obtained from animal after 3-day treatment with ortisone. A, B = rarified bright areas with some granules showing granulolysis. ×10,500

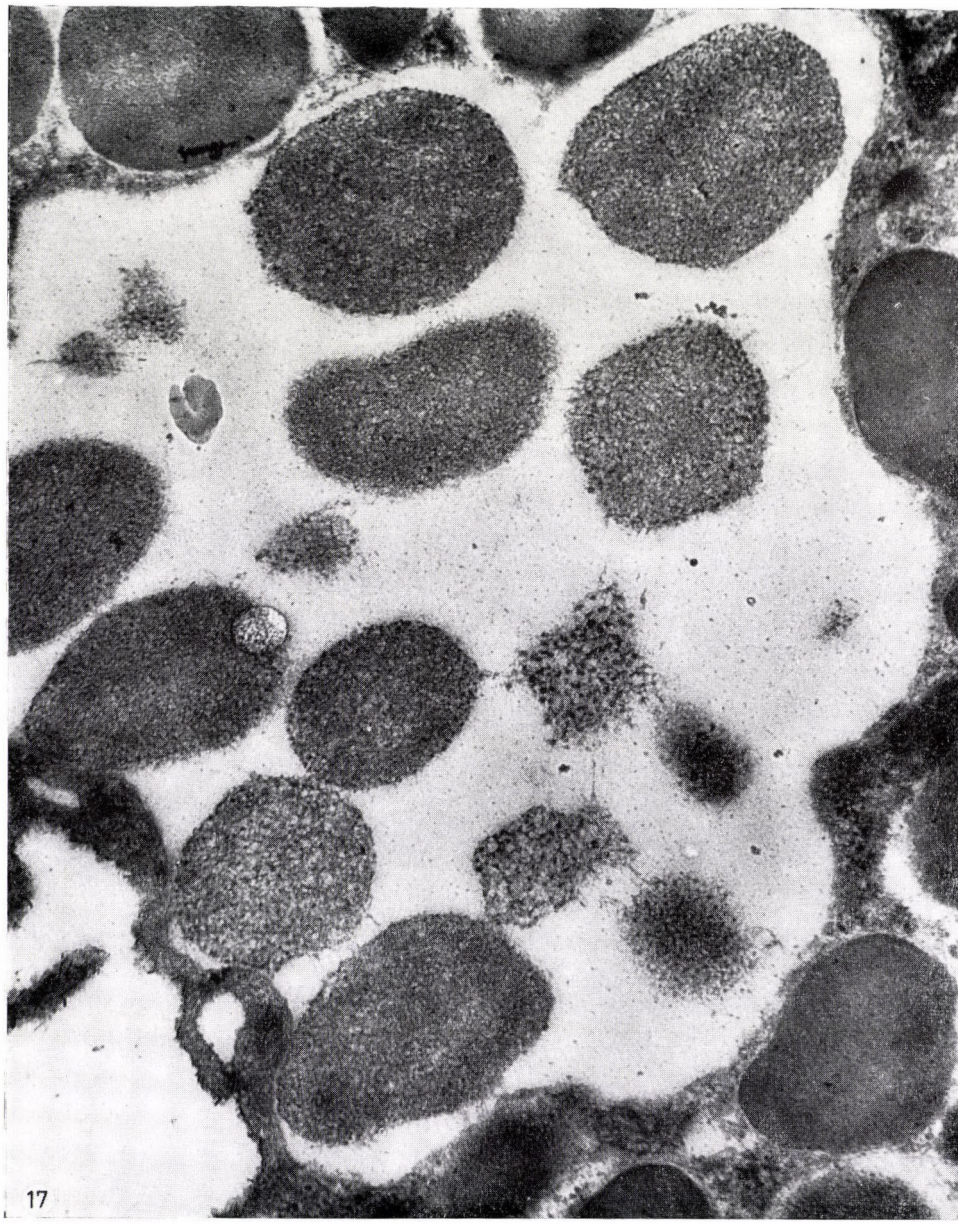


Fig. 17. High-power magnification of area A in Fig. 16. Granulolysis is well-visible. ×40,000

Uptake of $Na_2{}^{35}SO_4$ by peritoneal mast cells

Quantitative results are listed in Table 2. The safranin component of the dye had evidently been eluted from the autoradiographic preparations during development and fixation, whereas the cells reacting with alcian blue were clearly visible. Massive precipitation of silver grains was obtained at the site or in the immediate vicinity, of most of the cells (Fig. 13).

Electron-microscopic examination of peritoneal mast cells

The ultramicroscopic pattern of peritoneal mast cells in the control animals was as described in literature [14, 27, 29]. Some cells showed the normal structure in the test animals also (Fig. 14), with the only difference that the number of processes projecting from the cell surface was conspicuously increased. These processes (Fig. 15 : p) were presumably formed during the disappearance of the granules (Fig. 15 : g) and may have represented the remnants of detached vacuoles (Va_1 and Va_2), i.e., exocytotic structures. The granules of the cells were of different densities, with mitochondria (mi) between them. It was often possible to follow the process of degranulation (Fig. 16): bright areas of reduced electron density, sharply circumscribed by the surrounding more electron-dense matrix, were seen in certain parts of the cytoplasm (Fig. 16 : A and B). Granules contained in them displayed the typical picture of granulolysis; the limiting membrane was loosened or had disappeared, and the granular substance had become less electron-dense (Fig. 17).

Discussion

The first and perhaps most significant conclusion to be drawn from the results of the present experiments is that thymectomy causes a change in the number of the peripheral (peritoneal) mast cells. Although earlier experiments [7—10, 12, 14, 21, 22, 25, 26] had proved that the thymus was involved in the genesis of mast cells, its influence on peripheral mast-cell formation had remained obscure. The present experiments seem to justify the assumption that thymectomy causes considerable decrease in the number of young lymphoid forms and a simultaneous increase in that of degenerating mast cells. The fact that no significant change occurred in columns 2 and 3 of the middle zone in Table 1, proves that thymectomy did not interfere with the continued maturation of young mast cells. It seems that the production of new cells was arrested while mature cells underwent degranulation. The whole picture was, thus, shifted to the right. This is supported by the fact that, though the differences owing to the high standard deviation were not significant, the number of cells in group 3 rose from 30 to about 50%.

The gradual disappearance of intact mast cells and their replacement by disintegrated ones in the cortisone-treated animals is in accordance with literary data [3, 26, 30, 35] and our earlier observations [11, 12, 14]. Certain authors affirm that cortisone destroys the mast cells [1, 4, 23, 28], while — according to others — it increases their number [12, 14, 26] or has no effect on them [6, 28]. When estimating the effect of cortisone on the mast cells, we have to take into account the site where the mast cells are examined. At a time when the destruction of peritoneal mast cells is most intensive, a conspicuously high number of young forms appear in the thymus and in the lymph nodes. It can therefore be correlated that *cortisone promotes the formation of mast cells at the site of their genesis, but accelerates their maturation and causes their degranulation (decomposition) at the periphery*. This is to some extent in agreement with our earlier experiments [11] in which locally applied prednisolone first increased and then decreased the number of mast cells, and explains the effect of cortisone on mast cells.

As regards the acute effect of prednisolone, it would seem from the experiments that a shift to the right like that caused by cortisone fails to occur after treatment with prednisolone. It is probable that in the case of acute treatment the time is not long enough for either the destructive or the constructive tendency to get predominance. This is to say that, while also peripheral mast cells are capable of maturing, this process and that of decomposition following hypergranulation are in a sort of equilibrium so that it would depend on the arbitrarily chosen time of observation (an hour in the given case) in which phase one happens to observe the cells. Since treatment with cortisone lasted three days, we saw in this respect a picture of definitively stabilized conditions.

The situation is somewhat different if prednisolone is applied to thymectomized animals. Compared to the untreated controls (Table 1), shift to the right was more pronounced than after simple thymectomy. All normal cells decreased and only the damaged ones increased in number. The result was similar when the combined effect of thymectomy + prednisolone was compared with that of thymectomy alone or the combined effect of thymectomy + prednisolone with that of prednisolone alone. Since hormonal treatments followed thymectomy after a lapse of about 45 days, it is safe to assume that mast cells produced by thymectomized individuals are not quite normal and more sensitive to outside influence. This would support our earlier findings concerning mast cells produced in the lymph nodes of old animals (with involuted thymus) [10].

All these observations are, however, insufficient to elucidate the role of the thymus in the peritoneal phenomena observed in respect of mast cells. Changes produced by thymectomy may have been due to the removal of the very organ of mast-cell formation. It is, on the other hand, possible that the

thymus synthesizes a substance which increases the resistance and influences the maturation of mast cells. It might be supposed that the observed changes were caused by the absence of this substance. However, the fact that *mast-cell formation is in progress in the thymus while mast cells are being degranulated at the periphery* admits of the inference that it is the *removal of the thymus, the producer of mast cells*, which induces decrease in the number of young forms and degranulation (disintegration) of mature cells. This assumption seems to be justified if we consider that mast cells of the first category are of lymphoid character and can, therefore, be supposed to have been mostly originated from the thymus.

It is by the present authors that young mast cells were first described from adult animals. COMBS and co-workers [5] described such young forms as representing a certain phase of development, but they observed these lymphoid forms only in quite young animals, and found merely safranin-positive cells in adult ones. We too observed only such cells in the subcutaneous tissue of adult animals, whereas also small lymphoid, alcian blue-positive mast cells were found by us in adult thymus and lymph node tissues. It is the number of these cells which grows considerably under the influence of glucocorticoids. Representing a phase in the development of mast cells, they are identical with the cells stained with aldehyde fuchsin and identified also electron-microscopically in our earlier experiments [14].

The next problem arising from the present experiments is the mechanism of action of glucocorticoids. It was demonstrated in earlier experiments that glucocorticoids were incorporated in the granules of mast cells [13, 15], and our model experiments [16] have allowed the conclusion that they facilitate the binding of histamine and heparin, the two main components of granules. The model experiments proved histamine and serotonin rather than heparin to be the primary components of mast-cell granules, although heparin too is indispensable for their composition. Also the present fluorescence-microscopic experiments have shown that cortisone increases the number of cells containing serotonin (or possibly histamine). The number of these cells was found to be considerably higher than of those demonstrable by the extremely sensitive staining with alcian blue-safranin, i.e., of the cells that can be regarded as mast cells on the evidence of light-microscopy (Figs 11, 12). Prolonged cortisone treatment increases the number of such cells in the peritoneum, thymus and lymph nodes alike. This phenomenon was especially conspicuous in the thymus, where the majority of reticular cells contained serotonin as soon as after three days of cortisone treatment. It follows from the foregoing that there exist many cells in the thymus which — while containing a component of mast-cell granules — cannot be regarded as mast cells (at least not according to current terminology) but are able to produce mast cells or are themselves potential mast cells. Such cells are of the lymphoid type and occur also in the peritoneal fluid.

It has been assumed that it is histamine fluorescence that is demonstrable after 24 hours of fixation in paraformaldehyde; the present experiments do not enable us to reject or confirm this supposition. What we actually observed was in increased fluorescence after 24 hours. However, the difference between tests and controls was the same, no matter whether paraformaldehyde fixation lasted one or 24 hours.

Besides promoting serotonin production, cortisone facilitates the incorporation of ^{35}S indicating acid mucopolysaccharide synthesis [17, 20, 24, 31, 34]. Peritoneal mast cells of the control animals took up hardly ^{35}S: its amount was the same in the supernatant fluid as in the cells, whereas the cells of cortisone-treated animals contained twice as much sulphur than did the supernate, a sign of considerable sulphur uptake. Quantitative results obtained in Bürker's chamber by staining with toluidine blue showed the number of mature — toluidine-positive — mast cells to have increased after treatment with cortisone. Comparison of isotope measurements with the results of mast-cell count reveals that, while cortisone treatment increased the number of toluidine-blue metachromatic mast cells about threefold, the amount of ^{35}S incorporated by the cells was four times that taken up by the controls ($3.58\times10^3-3.11\times\times10^3 = 0.47\times10^3$; $3.31\times10^3-1.61\times10^3 = 1.70\times10^3$). This would mean that ^{35}S was taken up also by cells that did not react with toluidine blue. Considering that alcian blue-safranin staining was not feasible in Bürker's chamber, where only toluidine blue could be used, considering further that alcian blue makes visible also cells which fail to stain with toluidine blue, it is safe to assume that the surplus represented alcian blue-positive cells. This is supported by the fact that a significant precipitation of silver grains was observed above the alcian-blue-positive cells in autoradiographic preparations. It is probable moreover that the degree of ^{35}S incorporation was still higher because — as evident from the electron-micrographs — cortisone promotes degranulation. It follows that with the isotope technique just a phase of the dynamic process between uptake and excretion was registered; uptake was yet much more significant than in the controls.

Degranulation caused by cortisone seems to be very similar to the phenomenon caused by other "discharger" substances described in literature [27].

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BEITRÄGE ZUM MECHANISMUS DER MASTZELLENBILDUNG

Die Wirkung von Glykokortikoiden auf die Mastzellen normaler und thymektomisierter Ratten

Mit Hilfe von histochemischen, fluoreszenz- und elektronenmikroskopischen, Isotopen- und mathematisch-statistischen Untersuchungsmethoden wurde festgestellt, daß bei Ratten 1. nach Thymektomie in der Peritonealflüssigkeit die Zahl der jungen Mastzellen abnimmt, d. h., daß dem Thymus in der Mastzellenbildung eine Rolle zukommen kann, 2. auf Wirkung einer Dauerbehandlung mit Glykokortikoiden die peritonealen Mastzellen destruiert werden, bei gleichzeitigem Anstieg der Zahl der jungen Mastzellen im Thymus und in den Lymphdrüsen. Parallel läßt sich im Thymus, in den Lymphdrüsen und in der Peritonealflüssigkeit eine bedeutende Erhöhung der Anzahl der Serotonin-Fluoreszenz aufweisenden Zellen beobachten. Der Mechanismus der Mastzellenbildung sowie die Rolle der Thymusdrüse und der Glykokortikoide in diesem Prozeß werden besprochen.

ДАННЫЕ К МЕХАНИЗМУ ОБРАЗОВАНИЯ ТУЧНЫХ КЛЕТОК

Действие гликокортикоидов на тучные клетки нормальных и тимектомированных крыс

Результаты гихтохимических, флюоресцентно- и электронномикроскопических радиоизотопных и математико-статистических методов исследования показали, что у крыс 1) после тимектомии в перитонеальной жидкости понижается число молодых тучных клеток; следовательно, зобная железа, быть может, играет роль в образовании тучных клеток; 2) под влиянием длительной дачи гликокортикоидов происходит разрушение перитонеальных тучных клеток; в то же время в зобной железе и в лимфатических узлах увеличивается число молодых тучных клеток. Одновременно в зобной железе, в лимфатических узлах и в перитонеальной жидкости наблюдается значительное повышение числа клеток, показывающих серотонин-флюоресценцию. Обсуждается механизм образования тучных клеток и роль зобной железы и гликокортикоидов в этом процессе.

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L-GLUTAMINE: D-FRUCTOSE-6-PHOSPHATE AMINOTRANSFERASE (EC 2.6.1.16) ACTIVITY IN MAMMALIAN TISSUES

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Synopsis

L-glutamine: D-fructose-6-phosphate aminotransferase activity was determined in the liver, kidney, lung, intestine, brain, and heart muscle of the guinea-pig, dog, pig, calf, ox, and white rat as well as in several regions of the gastrointestinal tract of pig. The highest activity was found in the intestine of the guinea pig, dog, and in the liver of the guinea-pig and rat. In the gastrointestinal tract of pig, the duodenum has the highest aminotransferase activity. Certain suggestions are offered as regards the significance of aminotransferase in the biosynthesis of mucopolysaccharides.

Introduction

Mucopolysaccharides and glycoproteins are substances that are widely distributed by nature. There is a theoretical possibility of synthesis of the glucosamine-6-phosphate, aminosugar precursor, by one of four independently acting enzymes. Of these L-glutamine: D-fructose-6-phosphate aminotransferase [8], deaminase [9], and transaminase [12] synthesize them from unaminized sugars, and hexokinase by glucosamine phosphorylation [1].

Until now the relationship between these enzymes has not been investigated as regards their action in glucosamine-6-phosphate biosynthesis and the extent of the participation of any of them in this process. We have data concerning the participation of these enzymes in controlling the amount of synthesized aminosugar precursor. KORNFELD [7] reported the existence of feedback inhibition of aminotransferase by UDP-GlcNAc.

In this paper we present our data on the distribution of the aminotransferase activity which, together with the results obtained by LELOIR and CARDINI [9], point to the possible presence of two differently acting enzymes controlling the glucosamine-6-phosphate biosynthesis. This paper was presented in part at the III. F. E. B. S. conference [15].

Material and method

L-glutamine was obtained from the Koch-Light Laboratories, the barium salts of glucose-6-phosphate and fructose-6-phosphate from Boehringer Werke, gelatine from the Gelatine Factory in Pulawy, Poland.

The barium salts, glucose-6-phosphate and fructose-6-phosphate were converted to potassium salt by means of ion exchange resin Dowex-50 [4]. The rat, guinea-pig, and dog organs were obtained immediately after decapitation of the animals, the organs of the other animals were obtained from slaughter-house, and transported to the laboratory in containers at 0—4 °C.

The enzyme was isolated at a temperature of 0—4 °C, and extracted as follows:

Procedure A. 1 g of each tissue, finely cut, was homogenized in 4 ml of an ice-cold solution containing KCl 0.154 *M*, EDTA (ethylenediamine tetraacetate) 0.001 *M*, glucose-6-phosphate 0.012 *M* pH 7.2 (14), in a motor-driven homogenizer with a teflon pistle for 20 sec. The homogenate was centrifuged for 90 minutes at 7,000 xg.

Procedure B. 2 g of each tissue, finely cut, was homogenized in 8 ml of an ice-cold solution KCl 0.154 *M* and EDTA 0.001 *M* pH 7.2 in the same homogenizer for 40 sec. The homogenate was centrifuged for 90 min. at 7,000 xg.

Procedure C. 2 g of each tissue, finely cut, was homogenized in 8.0 ml of ice-cold phosphate buffer 0.1 *M* pH 7.5, containing 0.004 *M* EDTA, in a motor-driven homogenizer for 40 sec. The homogenate was centrifuged for 60 min. at 12,000 xg.

In procedures A, B and C the supernatant was the source of the enzyme.

Enzymatic activity was determined by the routine method as follows: 1.5 ml of the reaction mixture consisted of 1 ml of an incubating fluid containing 0.012 *M* fructose-6-phosphate, 0.012 *M* L-glutamine, 0.001 *M* EDTA and 0.05 *M* phosphate buffer, pH 7.7 in procedure A; 0.02 *M* fructose-6-phosphate, 0.012 *M* L-glutamine, 0.001 *M* EDTA, and 0.05 *M* phosphate buffer pH 7.7 in procedure B; 0.01 *M* glucose-6-phosphate, 0.012 *M* L-glutamine, 0.004 *M* EDTA, 0.1 *M* phosphate buffer pH 7.5 in procedure C, and 0.5 ml of the supernatant as a source of the enzyme. After 60 min. incubation at 37 °C, the reaction was stopped by heating the incubated mixture for 2 minutes. The glucosamine-6-phosphate obtained was determined by the method of REISSIG and co-workers [16] according to GHOSH and co-workers [5], glucosamine hydrochloride being used as a standard. In all cases blank tests were carried out by adding 0.5 ml supernatant to 1 ml of the incubation mixture with fructose-6-phosphate or glucose-6-phosphate, heated to 100 °C, and heating the fluid for further 2 minutes. All the activity determinations were carried out on double parallel samples.

The activity of D-glucose-6-phosphate ketol-isomerase in homogenates was detected by the method previously described [18].

Protein was determined by the biuret GLEISS and HINSBERG method [11] using gelatine as a standard. The gelatine standard was prepared by dissolving 2 g gelatine in 100 ml 0.154 *M* KCl, 0.001 *M* EDTA pH 7.2 in procedure A and B, and 0.1 *M* phosphate buffer, 0.004 *M* EDTA pH 7.5 in procedure C, at about 50 °C. The fluid was kept in portions of about 5 ml. at a temperature of —25 °C. The frozen standards were heated to 40—50 °C just before determination.

Results

D-glucose-6-phosphate ketol-isomerase activity. After incubation of both glucose-6-phosphate and fructose-6-phosphate separately with the supernatant, a mixture of glucose-6-phosphate and fructose-6-phosphate was obtained. The products of D-glucose-6-phosphate ketol-isomerase reaction were separated by chromatography and identical patterns were obtained, regardless of whether glucose-6-phosphate or fructose-6-phosphate was used as substrate. In both cases the spots of glucose-6-phosphate were saturated to a greater degree than the fructose-6-phosphate spots.

Aminotransferase activity in animal tissues. The activity of L-glutamine: D-fructose-6-phosphate aminotransferase in animal tissues, expressed in μM glucosamine-6-phosphate formed in 1 hour by the supernatant fluid from 1 g wet tissue is presented in Table 1.

As shown in Table 1, more than 2.0 μM glucosamine-6-phosphate is synthesized in 1 hour by the supernatant fluid from 1 g of the intestine of the

guinea-pig, pig, and dog, while supernatant from the liver of the rat produced only 1.53 μM glucosamine-6-phosphate in the same conditions.

Table 1

L-glutamine: D-fructose-6-phosphate aminotransferase activity in mammalian tissues

The enzyme was extracted and determined according to procedure A. Enzymatic activities were expressed in μM glucosamine-6-phosphate formed in 1 g of wet tissue. In the table the mean values of 3 experiments are given

	Liver	Kidney	Lung	Intestine	Brain	Heart muscle
Guinea-pig	1.88	0.80	0.79	2.13	0.46	0.42
Pig	0.55	0.50	1.15	2.16	0.33	0.15
Dog	0.78	0.47	0.56	2.62	0.39	0.27
Calf	0.26	0.34	0.45	1.35	0.06	0.02
Ox	0.43	0.41	0.76	0.32	0.21	0.17
Rat	1.53	0.53	0.94	0.32	0.36	0.37

Table 2

L-glutamine: D-fructose-6-phosphate aminotransferase activity in mammalian tissues

The enzyme was extracted and determined as described for procedure A. Enzymatic activities are expressed in $\mu M \cdot 10^{-2}$ glucosamine-6-phosphate formed by 1 mg of protein of supernatant fluid. In Table 2 mean values of 3 experiments are given

	Liver	Kidney	Lung	Intestine	Brain	Heart muscle
Guinea-pig	2.7	1.2	1.2	5.8	3.2	1.1
Pig	0.7	1.3	1.9	4.9	2.3	0.4
Dog	1.0	0.8	0.7	5.7	1.6	0.7
Calf	0.3	0.6	1.2	3.5	0.3	0.05
Ox	0.5	0.7	1.5	0.8	1.1	0.5
Rat	1.5	0.9	1.2	0.8	1.6	0.7

Table 2 presents the aminotransferase activity as $\mu M \cdot 10^{-2}$ glucosamine-6-phosphate synthesized by 1 mg of protein of supernatant fluid. The highest, over $4.0 \cdot 10^{-2}$ μM, glucosamine-6-phosphate was produced by 1 mg of protein in supernatant fluid from the intestine of the guinea-pig, pig and dog, the least amount of glucosamine-6-phosphate was produced by the heart muscle of the calf.

Aminotransferase in gastrointestinal tract of pig. Pig intestine has a high activity in μM per 1 g wet tissue/hour, as well as in μM per 1 mg of protein/hour.

We have therefore established the distribution of activity in several regions of the gastrointestinal tract.

The results are presented in Tables 3 and 4.

Table 3

L-glutamine: D-fructose-6-phosphate aminotransferase activity in the gastrointestinal tract of pig

The enzyme was extracted and determined according to procedure B
In the table the mean values from 6 experiments are given

	Protein in mg in 1 ml of supernatant fluid	Hexosamine formation	
		in $\mu M \cdot 10^{-2}$ per 1 mg of protein	in μM per 1 g of wet tissue
Esophagus	5.12 ($\pm$1.1)	1.72 ($\pm$0.71)	0.33 ($\pm$0.13)
Stomach	7.54 ($\pm$2.2)	5.1 ($\pm$3.28)	1.23 ($\pm$0.88)
Duodenum	9.25 ($\pm$0.94)	5.23 ($\pm$5.13)	1.94 ($\pm$1.89)
Small intestine	9.1 ($\pm$1.37)	4.23 ($\pm$2.58)	1.53 ($\pm$0.9)
Large intestine	6.3 ($\pm$1.0)	4.4 ($\pm$2.17)	0.91 ($\pm$0.34)

Table 4

L-glutamine: D-fructose-6-phosphate aminotransferase activity in the gastrointestinal tract of pig

The enzyme was extracted and determined according to procedure C
In the table the mean values from 6 experiments are given

	Protein in mg in 1 ml of supernatant fluid	Hexosamine formation	
		in $\mu M \cdot 10^{-2}$ per 1 mg of protein	in μM per 1 g of wet tissue
Esophagus	6.21 ($\pm$0.8)	19.14 ($\pm$15.8)	4.83 ($\pm$4.08)
Stomach	8.75 ($\pm$1.35)	16.96 ($\pm$ 4.46)	6.98 ($\pm$2.04)
Duodenum	12.0 ($\pm$1.67)	40.52 ($\pm$19.6)	19.25 ($\pm$8.95)
Small intestine	12.83 ($\pm$4.41)	16.88 ($\pm$ 5.23)	8.65 ($\pm$2.88)
Large intestine	8.67 ($\pm$1.38)	20.32 ($\pm$10.8)	7.47 ($\pm$5.48)

Table 3 presents the results of determinations of the enzyme activity from several regions of the pig gastrointestinal tract in homogenization medium without buffer (0.154 *M* KCl, 0.001 *M* EDTA), and in Table 4, the activity of the enzyme extracted in 0.1 *M* phosphate buffer with 0.004 *M* EDTA pH 7.5 is shown.

Comparison of the data of Tables 3 and 4 show, that in enzyme preparation extracted from tissue in 0.1 *M* phosphate buffer a 5 to 10 times higher activ-

ity, calculated per 1 g of wet tissue, and a 4 to 8 times higher activity per 1 mg of protein was observed.

To determine the reason for the low activity of enzyme extracted in KCl without a buffer, and the high activity in 0.1 *M* phosphate buffer extracts, the pH of each supernatant fluid, and the pH of each incubation mixture before and after the 60 min. incubation period were measured. Determination of enzymatic activity was repeated after 7 hour storage of each extract in +4 °C in order to evaluate the influence of the phosphate buffer on the stability of the enzyme. Table 5 shows the results of this investigation.

Table 5

The influence of pH and 0.1 M phosphate buffer on the stability of L-glutamine: D-fructose-6-phosphate aminotransferase activity

The enzyme was extracted from the pig small intestine. In experiments 1—4 the extraction was performed according to procedure B, and in experiments 5—8 according to procedure C. Each of the homogenates was centrifuged for 60 min. at 12,000 xg. The enzymatic activity was determined by incubation of 0.5 ml of each supernatant fluid with 1 ml incubation mixture consisting of: fructose-6-phosphate 0.005 *M*, L-glutamine 0.006 *M*, phosphate buffer 0.05 *M*, EDTA 0.002 *M*, pH 7.5 in exp. 1—4; fructose-6-phosphate 0.005 *M*, L-glutamine 0.006 *M*, phosphate buffer 0.1 *M*, EDTA 0.004 *M*, pH 7.5 in exp. 5—8. In exp. 1a—8a the determinations of enzymatic activity were performed after 7 hour storage of the supernatant fluids at a temp. +4 °C

No exp.	pH			Protein in mg in ml of supernatant fluid	Hexosamine formation		Remaining activity in per cent
	Supernatant fluid	Incubation mixture			in $\mu M \cdot 10^{-2}$ per 1 mg of protein	in μM per 1 g of wet tissue	
		before	after				
		60 min. incubation					
1	6.6	7.4	7.4	14.5	0.9	0.5	
1a					0.0	0.0	0.0
2	6.6	7.4	7.4	13.5	0.8	0.4	
2a					0.0	0.0	0.0
3	6.7	7.4	7.4	13.0	3.0	1.5	
3a					0.0	0.0	0.0
4	6.6	7.4	7.4	13.0	1.9	1.0	
4a					0.0	0.0	0.0
5	7.2	7.4	7.4	14.25	6.1	3.5	
5a					1.4	0.8	23.0
6	7.2	7.4	7.4	14.0	8.1	4.5	
6a					2.4	1.3	30.0
7	7.2	7.4	7.4	10.75	17.9	7.7	
7a					5.1	2.2	28.0
8	7.2	7.4	7.4	13.5	12.9	6.9	
8a					5.8	3.2	45.0

The results in Table 5 show that the pH of each incubation mixture was 7.4, and was not changed in the 60 min. incubation period. After 7 hour storage at +4 °C, the 0.1 *M* phosphate buffer extracts retained 23—45% of the enzymatic activity, whereas in KCl extracts the activity of the enzyme fell to 0.

Discussion

Łangowska and Roth [10] have reviewed many substances where glucosamine is an important constituent. Among them mucoitine sulphuric acid from the gastrointestinal mucus is a substance which protects the mucous membrane against mechanical damage and autodigestion. Supporting functions in skeletal tissues are performed by chondroitin sulphuric acid in the cartilage, chitin in the armour of insects, and murein in the bacterial cell wall. The glucosamine and its derivatives are constituents of many biological substances such as heparin, blood group substances or an intrinsic factor. From literature it can be seen that greater attention has been paid recently to the sugar and amino-sugar composition of glycoproteins.

Three enzymes have been described, which are capable of glucosamine synthesis. In 1953, Leloir and Cardini [8] found L-glutamine: D-fructose-6-phosphate aminotransferase which catalyzes reaction:

fructose-6-phosphate + glutamine → glucosamine-6-phosphate + glutamate

in extracts of *Neurospora crassa* and partially purified this enzyme. In 1960 Ghosh and co-workers [5] proved that the enzyme irreversibly synthesized glucosamine-6-phosphate.

The results reported by Leloir and Cardini [9] suggested that deaminase catalyzing reaction:

$$\text{glucosamine-6-phosphate} + H_2O = \text{fructose-6-phosphate} + NH_3$$

reversibly cleaves glucosamine-6-phosphate into fructose-6-phosphate and NH_3 in tissues. Later other authors [3, 17] presented data to show that deaminase plays an important role in the synthesis of glucosamine-6-phosphate.

The enzyme catalyzing reaction:

$$\text{glucosamine} + \alpha\text{-keto-glutaric acid} = \text{fructose} + \text{glutamate}$$

which reversibly cleaved glucosamine in the presence of α-keto-glutaric acid into fructose and glutamate was described by Pandolfo and Macaione [12]. These authors, however, stated that they managed to obtain a reverse reaction, a fact which may be of some significance as regards the biosynthesis of glucosamine.

In extracts from the mucous membrane of the pig gastrointestinal tract, we have confirmed by means of chromatography the presence of D-glucose-6-phosphate ketol-isomerase and thus in order to determine aminotransferase

fructose-6-phosphate activity in the alimentary canal we used glucose-6-phosphate or fructose-6-phosphate as a substrate.

We confirmed enzymatic activity in supernatant fluid after the sedimentation of nuclear and mitochondrial fractions. This would indicate that the cytoplasmatic-microsomatic fractions are the site of the biosynthesis of glucosamine-6-phosphate, a finding in agreement with the reports of other authors [6, 13].

The high activity of L-glutamine: D-fructose-6-phosphate aminotransferase in the supernatant fluid could be the result of high content of aminotransferase, low deaminase activity or low concentrations of natural inhibitors (UDP-NAcGlc). CLARKE and PASTERNAK [2], in extracts from *Bacillus subtilis* found both enzymes present, and separated aminotransferase from deaminase activity. Confrontation of the results of LELOIR and CARDINI [9], concerning deaminase activity, with the results concerning aminotransferase activity given in Tables 1 and 2 shows that high activities of aminotransferase are accompanied by a low deaminase activity. The reversely proportional correlation between aminotransferase and deaminase would seem to indicate that these two enzymes control the biosynthesis of glucosamine-6-phosphate, and subsequently mucopolysaccharides and glycoproteins.

Since a high activity per 1 g of tissue as well as 1 mg of protein was noted in the pig intestine, a study has been made in order to determine the distribution of aminotransferase activity in several regions of the gastrointestinal tract. As it can be seen from the results presented in Tables 3 and 4, the duodenum has the highest glucosamine-6-phosphate biosynthesis.

Comparing the results given in Tables 3 and 4 it follows, that the enzyme extracted in 0.1 *M* phosphate buffer have a several times higher activity than the enzyme extracted in 0.154 *M* KCl. This may indicate the influence of phosphate ions and pH on aminotransferase activity.

The results given in Table 5 show, that the low activity of the enzyme extracted from the small intestine of the pig in homogenizate without buffer does not depend on the change in pH during the 60 min. incubation period. The data given in Table 5 suggest that 0.1 *M* phosphate buffer has a stabilizing effect on the enzymatic activity in extracts of the pig small intestine. It is in agreement with the observations of one of the authors [19], which indicated that the phosphate buffer pH 7.5 in concentrations of 0.1 *M* or higher in the homogenizates and the incubations fluids, was necessary to protect the optimal conditions for the determinations of enzymatic activity.

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L-GLUTAMIN: D-FRUKTOSE-6-PHOSPHAT-AMINOTRANSFERASEN-AKTIVITÄT (E.C. 2.6.1.16) IN SÄUGETIERGEWEBEN

Die Aktivität von L-Glutamin: D-Fruktose-6-phosphat-Aminotransferase wurde in der Leber, Niere, Lunge, im Darm, Gehirn und Myokard von Meerschweinchen, Hund, Schwein, Kalb, Rind und weißer Ratten ferner in den verschiedenen Abschnitten des Magen-Darm-Traktes von Schweinen bestimmt. Die höchste Aktivität wurde im Darm von Meerschweinchen, Hund und Schwein sowie in der Leber von Meerschweinchen und Ratte nachgewiesen. Im Verdauungskanal des Schweins zeigt das Duodenum die höchste Aktivität. In bezug auf die Bedeutung der Aminotransferase in der Biosynthese von Mukopolysacchariden werden verschiedene Hypothesen vorgeschlagen.

L-ГЛЮТАМИН: D-ФРУКТОЗА-6-ФОСФАТ-АМИНОТРАНСФЕРАЗНАЯ АКТИВНОСТЬ (E.C.2.6.1.16) В ТКАНЯХ МЛЕКОПИТАЮЩИХ

Активность L-глютамина-D-фруктоза-6-фосфат-аминотрансферазы была определена в печени, почках, легких, кишечнике, мозге и миокарде морских свинок, собак, свиней, телят, рогатого скота и белых крыс, а также в различных отделах желудочно-кишечного тракта свиньи. Наивысшая активность была найдена в кишечнике морских свинок, собак и свиней, а также в печени морских свинок и крыс. Среди отделов желудочно-кишечного тракта свиньи наивысшую аминотрансферазную активность показывает двенадцатиперстная кишка. Авторы предлагают несколько предположений относительно значения аминотрансферазы в биосинтезе мукополисахаридов.

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CYTOMORPHOLOGICAL EFFECT OF FACTOR C IN SUBMERGED CULTURES ON THE HYPHAE OF STREPTOMYCES GRISEUS STRAIN NO. 52–1

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Synopsis

The cytomorphological effect of factor C, an endogenous initiator of differentiation in *Streptomyces griseus* has been studied on the hyphae of *Streptomyces griseus* strain No. 52—1 under conditions of submerged cultivation. In submerged culture the differentiation of this strain seemed to be retarted: no reproductive elements e.g. conidia were present that characterized strain No. 45H, the strain which produced factor C. Addition of factor C to the submerged culture of strain No. 52—1 was followed by a massive appearance of reproductive (conidium-producing) hyphae which showed the same cytomorphological pattern as that observed in the filaments of strain No. 45H. A microscopic method is described by which the various hyphal types, namely the vegetative, reproductive, degenerative and lysed forms are easily distinguishable.

Introduction

The cytomorphological picture of the hyphae of Streptomyces strains grown in liquid culture media undergoes certain regular changes with advancing age. The life cycle of *Streptomyces* can be divided into clearly distinct phases on the evidence of morphological and biochemical changes [3]. The life cycle starts with reproductive forms, passes through the germinative, vegetative and reproductive phases of growth, and terminates with the renewed formation of reproductive elements. The ways in which reproductive structures are produced in submerged cultures are shown in the scheme elaborated by PÉNAU [2].

Comparison of streptomycin-producing *Streptomyces griseus* strain No. 52—1 with the streptomycin-nonproducing strain No. 45H shows a difference not only in the duration of their respective life cycles but, as evidenced by their cytomorphological features, the two strains even follow different paths of PÉNAU's scheme. Under the conditions of submerged culture, strain No. 52—1 being retarded its reproductive phase, does not reach the conidial stage of reproduction [10].

While the cytomorphological picture of the hyphae remains homogeneous during the germinative and vegetative phases of growth, it becomes heterogeneous in the reproductive phase: in addition to the existing vegetative filaments there appear hyphae developing into the reproductive type, the latter

represent different stages of differentiation and there appear also degenerative forms [9].

Strain No. 45H has a short life cycle which terminates with abundant conidium production; by the end of the reproductive phase, a substance called factor C by us accumulates in the fermentation fluid. Factor C is capable of starting a process of differentiation in the filaments of strain No. 52—1 [5]. The biochemistry of the mode of action of this factor was dealt with in an earlier communication [7]. The present study was designed to study from a cytomorphological angle the process of differentiation elicited by factor C.

Material and method

Preparation of spore suspension from Streptomyces griseus strain No. 52—1. Spores, grown on the surface of soy-bean-agar medium [4] at 27 °C were suspended by means of glass beads in saline after 7 days of cultivation. The spores were counted in Bürker's chamber. The spore suspension was stored at +4 °C for not longer than three weeks.

Cultivation and testing of the effeciency of factor C. Filtered soy-bean medium [4] was used for studying the effect of factor C. Each ml of the medium was seeded with 1.6 to 1.8 million spores. Incubation was performed on a rotating shaker (stroke 50 mm., 220 r.p.m.) at 27 °C. Three ml of the medium were placed in each of the 15 ml vials.

After sterilizing factor C by passing it through a glass filter G5, we diluted it to different degrees, and 0.3 ml of each dilution to be examined was then added to 3 ml medium simultaneously with the inoculation.

Sampling. Two parallel cultures per sample were used. To avoid errors due to differences in volume, cultivation was discontinued in the vials from which samples had been taken.

Fixing. One ml of the culture was withdrawn with a pipette; great care was taken to prevent mycelium, adhering to the wall of the tube, from passing into the sample (it has a different cytomorphology which would have vitiated the evaluation of results). One ml each of the mycelium suspension was then added in a centrifuge tube to 2 ml fixative at +4 °C for from 30 min. to 24 hours. The fixative was composed of 6% glutardialdehyde and 7.5% sucrose in M/15 PO_4 buffer at pH 7.2.

Preparation of slides. The fixed mycelium was centrifuged and washed three times with distilled water. At each washing, the washing fluid was left standing with the suspension at a temperature from 0 to +4 °C for at least 30 min. The sediment was finally suspended in distilled water, and a thin smear (as far as possible, of not more than one-cell thickness) was spread on a slide by squashing mycelial clumps with another slide. The preparations were dried in air.

Staining; microscopic examinations; demonstration of cell wall. Soaking for 15 min. in 0.5% EDTA solution adjusted to pH 7.0 by sodium hydroxide.

Washing with distilled water three times, for 5 min. each.

Soaking for 10 min. in 5% phosphomolybdenic acid (orange-yellow colour).

Washing with distilled water three times, for 5 min. each.

Steeping for 10 min. in a solution containing 20% tannin and 4% phenol.

Washing with distilled water three times, for 5 min. each.

Staining for 20 min. with a solution containing 0.05% toluidine blue, 0.05% crystal violet, 0.05% Janus green, 0.05% neutral red, 0.05% Bismarck brown, 0.5% NaCl in 0.1 *M* veronal acetate buffer of pH 5.5.

Washing with tap water until drippings were colourless, rinsing with distilled water, drying in air at room temperature.

Examination. A Reichert-Zetopan microscope, provided with Auflicht Fluorit oil-immersion objective ×100, was used. The diaphragms of the top-lit condenser were so adjusted as to cause the surface of the slide reflect a minimum of light. By suitable adjustment of Apl. 1,35 condenser beneath the object it was achieved that part of the light, passing through the object from above, was reflected and evenly illuminated the field of vision with slight intensity. In this illumination the wall appeared bluish-grey, the vegetative hyphae yellowish pink, the reproductive hyphae bright red and the lysed hyphae, yellowish white.

Polysaccharides were demonstrated by Schiff's periodic acid reaction [1] and studied

under a microscope of the Reichert-Zetopan type provided with Anoptral contrast equipment (PhA × 100 objective) and a green colour filter. Areas with positive reaction shone before the dark background in a yellowish-green colour depending on the intensity of the reaction.

Results and discussion

If added at the moment of inoculation, factor C had no effect either on the duration of the germinative phase or the number of germinative spores [6]. The cytomorphological picture of the spores was affected but slightly.

Microscopical examination revealed in the vegetative phase, by the 60 to 66th hour, no cytomorphological difference between the hyphae of the controls and those grown in the presence of factor C. The only physicochemical difference consisted at this stage in that the hyphae of the culture with factor C were clumped (like those seen in the culture of strain No. 45H), while no clumping occurred in the control culture [5, 10].

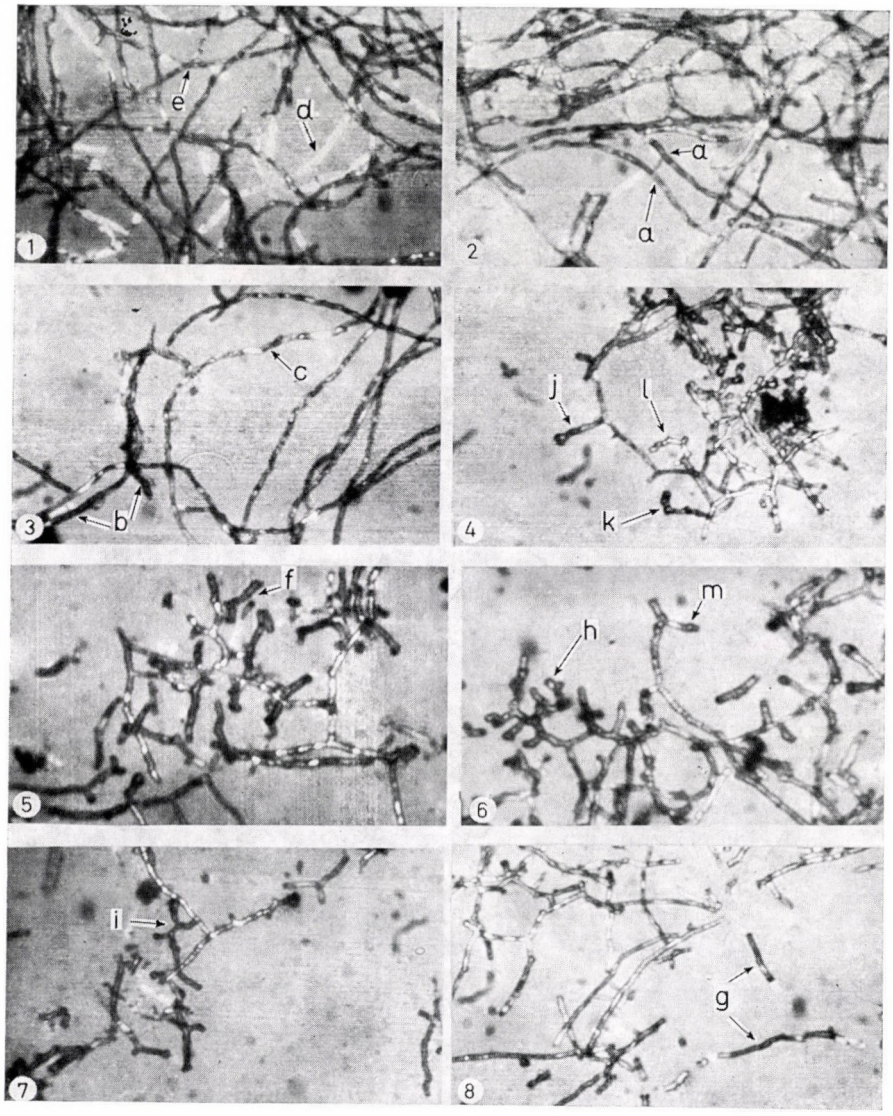
Cytomorphological differences became pronounced by 66 hours, and remained typical approximately until the 96th hour.

Wall staining showed in the controls (Figs 1, 2, 3) scantily ramifying, approximately straight vegetative (Fig. 2:a), early reproductive (Fig. 3:b) and degenerating (Fig. 3:c) filaments. Some hyphal segments displayed autolysis of variable degree (Fig. 1:d). Squared forms suggestive of arthrospores, were observed in the middle segment of certain hyphae (Fig. 1:e).

Adding of factor C (Figs 4 to 9) was followed by an increase in the proportion of thick-walled reproductive hyphae. Since these filaments are apt to break along the cross walls during the preparation of slides, numerous short fragments of reproductive hyphae were observed (Figs 5:f, 8:g).

Another characteristic effect of factor C consists in promoting the formation of side branches. Accordingly, many ramifying side branches termed C-branches by us were observed. (A typical such branch can be seen in Figs 6:h and 7:i). At first, the C-branches appeared as simple definitely reproductive side branches (Fig. 4:j). Provided the concentration of factor C was sufficient, the C-branches split into prespores and formed conidia, while their plasm retained the features characteristic of reproductive hyphae (Fig. 4:k). Some of the C-branches, though splitting likewise into prespores, degenerated as indicated by their staining resembling that of degenerated vegetative hyphae (Fig. 4:l). We succeeded in demonstrating these two C-branch types (reproductive and vegetative) also by Feulgen's reaction and by staining with methylgreen. The various nuclear types, their characteristics and significance will be published later.

Even the smallest effective dose of factor C induced the formation of reproductive branches with ramifying or simple bulging terminations. These branches were not straight, but rather frequently broken, the diameter of their parts frequently changed showing uneven trail (Fig. 6:m). When the dose of factor C was insufficient or the composition of the preparation was different



Figs 1—8. Wall staining. Combined illumination. ×1,300

Figs 1—3. Control. Hyphae from 70-hour submerged culture of *Streptomyces griseus* strain No. 52—1. a = vegetative; b = reproductive; c = degenerative; d = lyzed

Figs 4—6. Streptomyces griseus strain No. 52—1 growing in the presence of optimal concentration of factor C. Hyphae from 70-hour submerged culture. f = fragment of reproductive filament; h = typical C-branch splitting into spores; j = young reproductive side branch; k = sporulation in C-branch; l = degenerating C-branch with prespores; m = reproductive branches at the stage of incipient differentiation

Figs 7—8. Streptomyces griseus strain No. 52—1 in the presence of low concentrations of factor C. Hyphae from a 70-hour submerged culture. g = fragments of reproductive branches; i = C-branch not divided into prespores

only few cytomorphological features characteristic of the effect of factor C were seen. Fragmentation (Fig. 8) appeared to be the next stage of the effect. Higher (but still lower than the optimal) concentration of factor C elicited the appearance of C-branches which, however, did not divide into prespores (Fig. 7).

The minimum concentrations at which the crude preparations of factor C prepared in our laboratory were able to cause cytomorphological changes generally ranged between 40 and 150 μg/ml. Our hitherto purest preparation was effective in a concentration of 2 μg/ml.

PAS reaction. Employing Schiff's periodic acid reaction we demonstrated rod-shaped polysaccharides in 66- to 72-hour-old control filaments. The intensity of the reaction varied according to hyphal type. Polysaccharide distribution characteristic of the reproductive phase was rare [9], (Figs 9, 10).

Treatment with factor C slightly diminished the total amount of PAS-positive matter. The proportion of slightly and moderately positive hyphae on the one hand and strongly positive ones on the other had changed in favour of the former. Changes in distribution and not in quantity were, however, the principal phenomena characterizing the effect of factor C. The types with a distribution of PAS-positivity characteristic of the reproductive phase of strain 45H [10] and also degenerative forms appeared.

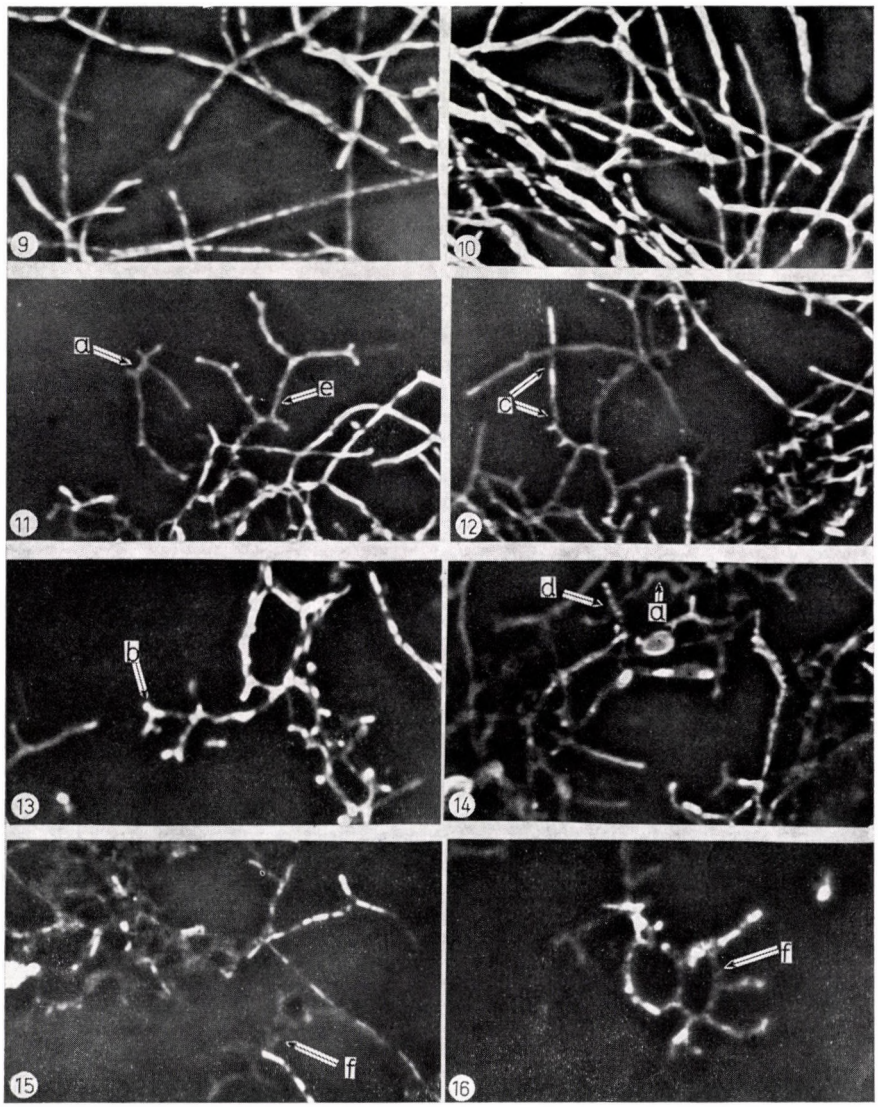
In order to facilitate orientation we divided the C-branches into four categories according to the intensity of PAS reaction:

Branches C I. The PAS reaction of these branches at the stage of incipient differentiation is very weak and perfectly homogeneous (Figs 11, 14:a). Comparison of wall staining and PAS reaction in the same cultures inclined us to regard these branches as transiting forms between the vegetative and the reproductive ones and partly as degenerative formations.

Branches C II. These are intensively PAS-positive at their termination, and gradually less positive farther from the end (Fig. 13:b). We regard them as young reproductive forms in which no fragmentation into spores has yet started. Part of them presumably undergoes subsequent degeneration.

Branches C III. PAS reaction is fairly strong along the cross walls, later also along the lateral walls. PAS-positivity is in accordance with the sporulated hyphal segments. (The arrow, marked "d" in Fig. 14, points to a young branch C III; Fig. 12:c shows the division of an intensively positive branch into prespores; Fig. 11:e presents the picture of a weakly positive branch beginning to develop into a branch C III). The frequency of branches C III is identical with that of C-branches with a definitely reproductive type wall staining, split into prespores.

Branches C IV. PAS positivity is seen around fully developed, rounded spores (Figs 15 and 16:f). Frequency is the same as that of the fully developed spore chains.



Figs 9—16. PAS reaction. Oil-immersion anoptral contrast examination. ×1,800

Figs 9—10. Control. *Streptomyces griseus* strain No. 52—1. Submerged culture at 70 hours

Figs 11—16. Streptomyces griseus strain No. 52—1 in the presence of different concentrations of the factor C. 70-hour culture. a = branch C I; b = branch C II; c = fragmentation of PAS-positive substance in intensively reacting filament (transition to C III); d = young branch C III; e = weakly PAS-positive branch C I (transition to C III); f = young branches C IV

Branches C I and C II belong probably to different lines of development: their differentiation follows separate ways. Differences in the intensity of the reaction in branches C III and C IV (which show at the same time an identical distribution of PAS positivity) may depend on whether the given more differentiated form is derived from branch C I or C II.

We succeeded but rarely in administering that optimal dose of factor C which causes a massive appearance of branches C IV.

In estimating potency of factor C preparations we regarded a preparation the more concentrated the larger number of highly differentiated forms appeared in its presence. (The effect was considered well pronounced if branches C II+ +C III exceeded 50% of the total number of hyphae).

The observed cytomorphological effect of factor C justifies the conclusion that it may influence the life cycle of *Streptomyces griseus* strains by initiating the reproductive phase of growth and/or by ensuring the normal course thereof. This would explain why the microscopic picture of the strain 52—1, if treated with factor C, becomes similar to that of strain 45H, whose life cycle is normal and short even in submerged cultures.

A series of experiments was carried out in order to ascertain the age at which cultures are most sensitive to factor C. The character of the cytomorphological effect and the time of its appearence were invariably the same if we added factor C to the medium between zero hour and 36 hours following inoculation, while no cytomorphological effect was observed if factor C was added at or after 48 hours. It follows that factor C acts only on cultures in an appropriate phase of the life cycle, and is unable to start a differentiation in senescent hyphae.

Biochemical investigations have moreover shown that cultures of *Streptomyces* respond to factor C only during the vegetative phase of growth [7]. Supposedly factor C acts by producing, directly or indirectly, a change in the activity of genes involved in the process of differentiation. Its effect depends, therefore, on the suitable loci being accessible to the influencing factor.

Further investigations concerning the effect of factor C are expected to yield additional information about other factors involved in the cytomorphological changes occurring in *Streptomyces* strains and, in general, about some of the principal characteristics of the internal programming in the process of differentiation.

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DIE WIRKUNG DES "FAKTORS C" AUF DIE CYTOMORPHOLOGIE DES STAMMES *STREPTOMYCES GRISEUS* NO. 52—1 BEI TIEFENFERMENTATION

Die cytomorphologische Wirkung eines auf die Differentiation wirkenden endogeneu Stoffes (im weiteren: Faktor C) auf die Fäden des Stammes *Streptomyces griseus* No. 52—1 wurde bei Tiefenfermentation untersucht. Die Differentiation des als Testobjekt herangezogenen Stammes No. 52—1 ist unter solchen Bedingungen gehemmt, es fehlen die bei dem große Mengen von Konidien entwickelnden, Faktor-C-produzierenden Stamm No 45 H beschriebenen reproduktiven Formen ähnlichen Gebilde. Auf Wirkung des Faktors C erscheinen auch in den Tiefenkulturen des Stammes No 52—1 große Mengen der reproduktiven (konidienbildenden) Fäden. Ihre Cytomorphologie ist analog der der Fäden des Stammes No 45 H. Ein Verfahren wurde erarbeitet, mit dessen Hilfe im mikroskopischen Bild die vegetativen, reproduktiven, degenerierenden und lysierten Fäden leicht voneinander zu unterscheiden sind.

ДЕЙСТВИЕ «ФАКТОРА С» НА ЦИТОМОРФОЛОГИЮ НИТЕЙ ШТАММА *STREPTOMYCES GRISEUS* № 52—1 ПРИ ГЛУБИННОЙ ФЕРМЕНТАЦИИ

Изучалось цитоморфологическое действие эндогенного вещества (в дальнейшем: «фактор С»), действующего на дифференциацию нитей штамма *Streptomyces griseus* № 52—1 в условиях глубинной ферментации. Дифференциация штамма № 52—1, используемого в качестве тест-объекта, при таких условиях оказалась заторможенной: отсутствуют образования, напоминающие репродуктивные формы, описанные в связи с штаммом № 45 Н, образующем большое количество конидий, производящих фактор С. Под влиянием фактора С в глубинных культурах штамма № 52—1 также появляются массы репродуктивных (образующих конидии) нитей. Цитоморфология последних аналогична той нитей штамма № 45 Н. Разработан метод, с помощью которого на микроскопической картине хорошо отличаются друг от друга вегетативные, репродуктивные, дегенерирующиеся и лизированные нити.

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CELL MIGRATION IN THE DEVELOPING FOLLICLE OF THE BURSA OF FABRICIUS BETWEEN CORTEX AND MEDULLA

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Synopsis

The development of the lymphoid follicle of the bursa of Fabricius was followed by light and electron microscope. On 13- to 18-day-old chicken embryos it was observed that the basement membrane of the epithelial bud was broken up at several sites. Young cells of mesenchymal character bearing cytoplasmic processes were frequently found in the discontinuity of the basement membrane. This phenomenon indicates significant cell migration between the cortical and medullary regions of the follicle during the mentioned period. The mesenchymal character and typically changed form of wandering cells and the behaviour of the basement membrane allow to conclude that the cells migrate from the cortical zone to the medullary region.

Introduction

The bursa of Fabricius, a thymus-like lympho-epithelial organ found exclusively in birds, arises as a dorsal diverticulum of the cloaca. The tunica propria of this organ is filled by lymphoid follicles which are closely related to the surface epithelium of the bursa [3, 17]. The follicles are divided by a basement membrane into a central medulla and a peripheral cortical zone [1, 2, 11]. Considering that the basement membrane separates cells of different origin, it is obvious that there are different cell populations at its two sides [4, 13].

The origin of the cell population of the follicles is very important, as the bursa plays a special function in the humoral immunity of birds [6, 7, 8, 10, 12, 16].

We have attempted to approach the development of the cell population of the follicle in the chicken embryos by light and electron microscope. In the course of these investigations, in the bursa of embryos 13—18-day-old we observed some significant cell wandering through the basement membrane between the cortex and the medulla.

In the present study we give a short account of this cell migration.

Material and method

White Leghorn hen eggs were incubated at 37 °C for 13 to 18 days. The developmental stages of the embryos were determined according to HAMBURGER—HAMILTON [9].

Half-thin sections were used and stained with warmed toluidine blue for light-microscopical investigations.

For electron microscopy, the removed bursa was cut into pieces of 1 cubic mm in 4% glutaraldehyde buffered with Millonig [14] and fixed for two hours at room-temperature. After a short washing the material was postfixed in buffered 1% osmium tetroxide. After dehydration the material was embedded in araldite. The thin sections were cut by a Reichert OMU—2 ultramicrotome. The ultrathin sections were counterstained with uranylacetate in 50% methanol [20] and with lead citrate [19], and examined in a JEM 6 C electron microscope.

Results

The bursa of Fabricius begins to differentiate in the chick embryo on the fifth day of incubation. The bursa first appears as an evagination of the cloaca, then grows into a vesicle on the ninth day of incubation. The inner surface of the bursa is plicated by the 11th day.

The beginning of the development of the follicle is indicated by some changes in the surface epithelium of the folds on the 13th day. At defined sites along the epithelium the epithelial cells exhibit a great mitotic activity and the epithelial bud projects into the tunica propria. The epithelial bud projecting into the underlying connective tissue pushes forward the basement membrane (Fig. 1). The undifferentiated cells located in the mesenchyma of the folds gather round the epithelial bud and begin to form the cortical region of the follicle.

As shown by electron micrographs these mesenchymal cells are generally oval or rounded-off, their nuclei being localized in the centre of the cytoplasm (Fig. 1). The chromatin substance of the nucleus is rather loose and appears generally in small clumps attached to the nuclear membrane. The smooth-surfaced endoplasmic reticulum and the Golgi complex are generally poorly developed. The abundance of free ribosomes in these cells is to be noted.

Such structural cells were observed in the opening of the basement membrane between the cortex and the medullary region (Figs 2, 3, 4). At the beginning these wandering cells insert into the opening by means of their processes. During this period the nucleus is eccentrically situated. Occasionally more than one cell are being in the opening (Fig. 2). At the same time it can be well observed that the edges of the basement membrane are bending inwardly to the epithelial bud.

The penetrating cell takes up a dumb-bell form. At the penetration site in the course of the penetration the cytoplasmic components (mitochondria and Golgi membranes) are perpendicularly oriented to the basement membrane (Fig. 3). The larger part of cell squeezes through the basement membrane while the discontinuity of the basement membrane becomes more and more pronounced. After the penetration of the nucleus the cytoplasmic processes of the epithelial cells approach each other and the opening of the basement membrane becomes narrower. Subsequently the hole is closed by the processes of epithelial cells, and thus the continuity of the basement membrane is restituted.

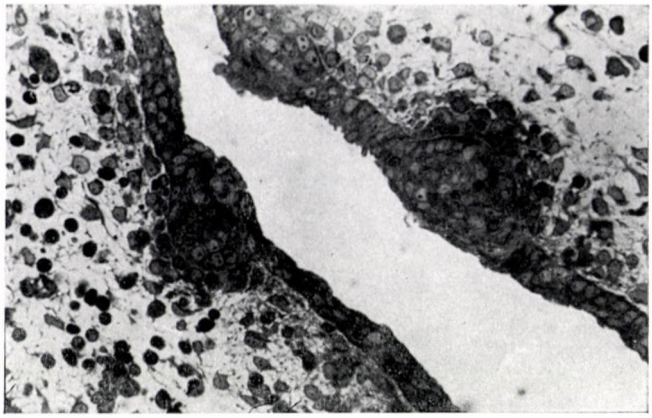

Fig. 1. Light micrograph, half-thin section. At the site of developing follicle the bulging epithelium projects into the underlying connective tissue. The undifferentiated mesenchymal cells gather round the developing follicle. Toluidine blue staining. Magnification: ×128

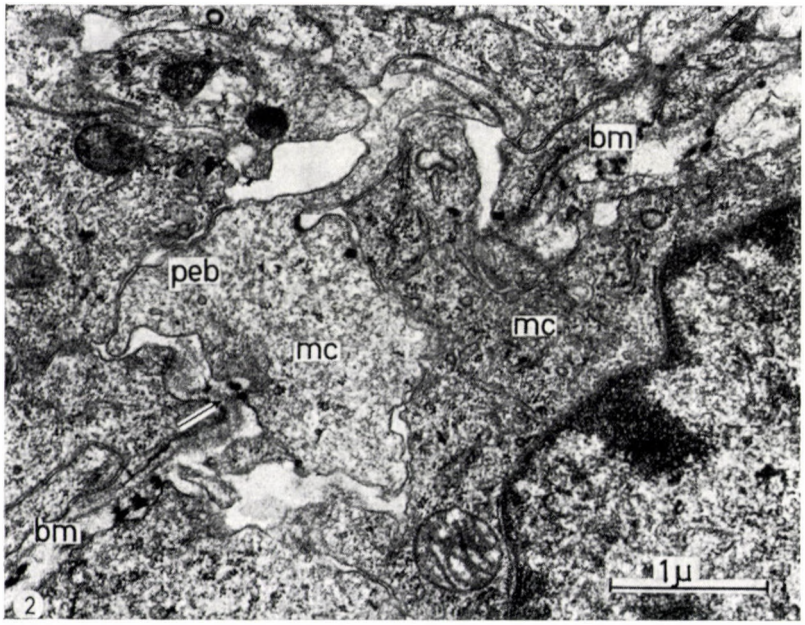

Fig. 2. Electron micrograph. Cytoplasmic processes of two migrating cells in an opening of the basement membrane (bm). Note that the edges of the ruptured basement membrane are bending inwardly to the epithelial bud (at index). Part of epithelial bud (peb); migrating cell (mc)

The migration begins on the 13th day and reaches the maximum activity on the 18th day. At this stage the basement membrane of a single follicle is broken at several, 4 or 5, sites at the same time, this may be seen on the sketch that was made of a montage (Fig. 5).

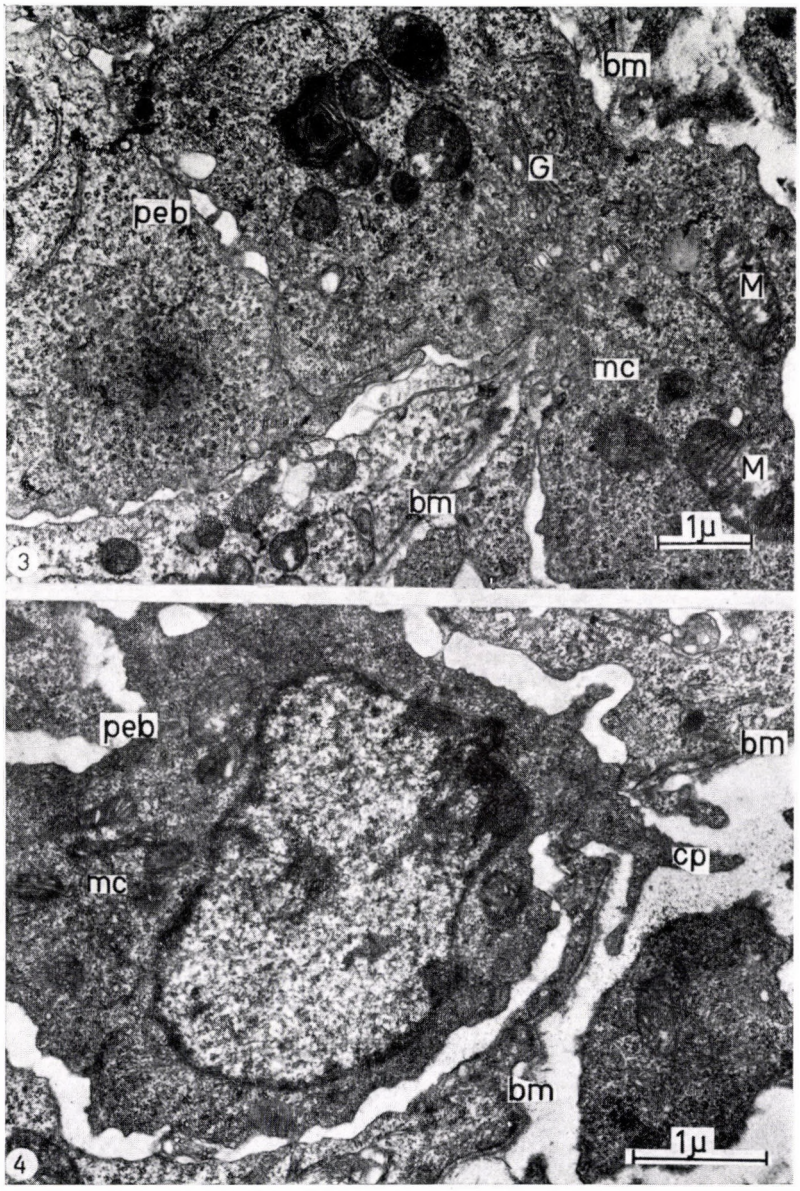

Fig. 3. Dumb-bell-shaped migrating cell in a discontinuity of the basement membrane (bm). The cytoplasmic components, Golgi complex (G) and mitochondria (M) are perpendicularly oriented to the basement membrane. Part of epithelial bud (peb). Migrating cell (mc)

Fig. 4. The final stages of penetration. The larger part of the cell is at the epithelial side of the basement membrane (bm). Note the bifurcated cytoplasmic processes (cp) at the other side of the basement membrane

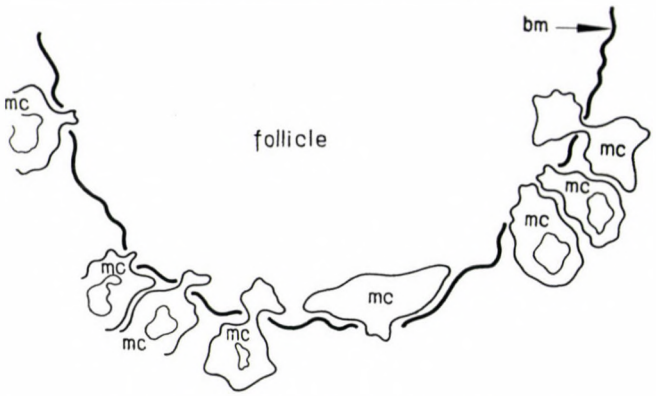

Fig. 5. A rough sketch making a montage of bursal lymphoid follicle from an 18-day-old chicken embryo. The basement membrane (bm) is broken at several sites. In the discontinuity migrating cells (mc) at different stages of penetration

Discussion

The first rudiment of the bursa of Fabricius appears as epithelial buds formed from the surface epithelium of folds. The appearance of epithelial buds are followed by gathering of mesenchymal cells round the bud probably due to the inductive capacity of the epithelial cells. When the cytoplasmic processes of a wandering cell reach the basement membrane, the membrane will be dissolved, probably by an enzymatic function of the cell, and a cytoplasmic process inserts in the hole thus formed [21].

Later the cell squeezes through the basement membrane, whereafter the hiatus of the basement membrane disappears. Naturally, static electronmicrographs cannot show the direction of this cell migration [5]. However, certain morphological marks are indicative of the direction.

The cell migration from the cortex forward to the medulla is established by the following facts:

(1) The migrating cells are similar to the cells that had been found in the tunica propria.

(2) The migrating cell becomes longer, its nucleus being located opposite to the cytoplasmic area inserting in the basement membrane.

(3) The edges of the ruptured basement membrane are bending inwardly to the epithelial bud. This bending is probably due to the mechanical effect of the migrating cell.

(4) Mesenchymal cells first appear at the border of the epithelial bud, and only somewhat later inside it.

Obviously these indirect data for the direction of migration do not contradict the statements of some authors that the lymphoid cells are derived by transformation of epithelial cells and only therafter migrate forward to the

cortical region [1, 2, 3]. But in the course of our investigation we could not observe signs of migration in this direction.

Both the mentioned morphological signs and experimental data of others refer to the migration of mesenchymal elements from the mesenchymal side into the epithelial bud and subsequent differentiation into mature lymphocytes [11]. MOORE and OWEN analyzed the origin of lymphoid cells in the developing bursa of Fabricius, using a chromosome marker technique in combination with various experimental procedures (parabiosis, twin-embryo, transplantation of bursal rudiments to the chorio-allantois and humoral modification of bursal development). These authors conclude that the lymphocytes of the bursa of Fabricius derive from blood-borne progenitor cells and these cells enter the bursal primordium during embryogenesis. Namely the mesenchymal cells deriving from other places in the organism gather round the epithelial bud and get into the primordium of the medullary region.

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ZELLENMIGRATION IN DEN SICH ENTWICKELNDEN FOLLIKELN DER BURSA FABRICII ZWISCHEN DER RINDENZONE UND DER MEDULLAREN REGION

Die Entwicklung der lymphoiden Follikeln der Bursa Fabricii wurde licht- und elektronenmikroskopisch untersucht. An 13 bis 18tägigen Hühnerembryonen wurde beobachtet, daß die Basalmembran der Epithelknospe an mehreren Stellen unterbrochen war. Junge Zellen mit Mesenchymcharakter, die zytoplasmatische Prozesse herbeiführten, fanden sich häufig an den Diskontinuität-Stellen der Basalmembran. Dies deutet an, daß im erwähnten Zeitpunkt zwischen der kortikalen und der medullaren Region der Follikeln eine signifikante Zellenmigration stattfindet. Der mesenchymale Charakter und der typische Formenwechsel der Wanderzellen sowie das Verhalten der Basalmembran erlauben die Schlußfolgerung, daß die Migration aus der kortikalen Zone in Richtung der medullaren Region vor sich geht.

МИГРАЦИЯ КЛЕТОК В РАЗВИВАЮЩИХСЯ ФОЛЛИКУЛАХ *BURSA FABRICII* ОТ КОРКОВОГО К МОЗГОВОМУ СЛОЮ

Развитие лимфоидных фолликулов bursa Fabricii было исследовано при помощи светового и электронного микроскопов. В 13 до 18-дневных зародышах цыплят непрерывность базальной мембраны эпителиальной почки была нарушена в нескольких местах. Молодые клетки мезенхимального характера, вызывающие цитоплазматические процессы, были часто обнаружены на местах нарушения непрерывности базальной мембраны. Это явление указывает на то, что в упомянутый период между корковым и мозговым слоями имеет место значительная миграция клеток. Мезенхимальный характер и типичное изменение формы странствующих клеток, а также поведение базальной мембраны позволяют заключить, что миграция направляется из корковой зоны к мозговому слою.

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HEXOSAMINE PRODUCTION IN VITRO BY RAT TISSUES UNDER AEROBIC AND ANAEROBIC CONDITIONS

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Synopsis

Comparative studies were performed on hexosamine production *in vitro* of minced rat liver, skin, kidney, spleen, as well of thigh and heart muscle in approximately anaerobic and aerobic environment. Using the method of POGELL and KOENIG the minced tissue was incubated for three hours at 37 °C, in the presence of glucose and glutamine and the tissue hexosamine was determined according to Boas' method. Well-demonstrable hexosamine production of variable degree was found in the liver, lung, skin, kidney and spleen, in 73—100% of the cases. Muscle tissue was found to be inactive. The extent of hexosamine production appeared to be dependent upon the oxygen supply of the tissue. In the liver hexosamine production was more intensive under anaerobic conditions in 12 cases, the opposite was observed in only one case. Anaerobic milieu appears to favour hexosamine production in the skin, kidney and lung tissues as well, while for the spleen the experimental results do not suffice for a conclusion in this regard.

Introduction

According to numerous data in literature, a correlation seems to exist between the mucopolysaccharide content of the tissues and their oxygen supply. Poorly vascularised tissues, such as cartilage, vitreous body, cornea, heart valve, have a low respiration and cytochrome oxydase activity, whereas they are rich in mucopolysaccharides [7, 13, 15].

Under pathological conditions, e.g., in tumours having a glycolytic metabolism with low respiration [25, 26] a high mucopolysaccharide level was observed [9]. KROMPECHER and co-workers [12] demonstrated that severe hypoxia is concomitant with high mucopolysaccharide content in varicose ulcer and in lung tuberculosis [3]. Similarly, a correlation was observed between poor vascular supply and deficient respiration and high mucopolysaccharide content of the tissue during cartilage neoformation by HADHÁZY and co-workers [10]. Investigations on phylogenetically lower animals living on land [14] and in the sea [19] demonstrated that high mucopolysaccharide level was associated with low cytochrome oxidase activity.

These data prompted us to investigate the origin of the excess mucopolysaccharide in the state of hypoxia, i.e., whether the locally accumulated muco-

polysaccharide is produced elsewhere (first of all in the liver) or may be produced *in loco* as well. The extrahepatic mucopolysaccharide production has been supported by numerous authors, more recently by BEKESI and co-workers [1] and BEKESI and WINZLER [2]. Increased mucopolysaccharide production was noted by SCHMIDT and co-workers [23] in experimentally strangulated rat skin in which hypoxia ensued in consequence of the mechanical occlusion of capillaries. On the basis of his investigations on fibroblast cultures, GROSSFELD [9] proposed a correlation between insufficient respiration, anaerobic metabolism and mucopolysaccharide content.

The mucopolysaccharide-hexosamine production *in vitro* by various tissues in anaerobic and aerobic milieu was also investigated.

The first observations on hexosamine production *in vitro* were published by LELOIR and CARDINI [16] on *Neurospora crassa*. CASTELLANI and ZAMBOTTI [8] demonstrated hexosephosphate-transaminase activity in epiphyseal cartilage homogenates and a less intensive activity in costal cartilage, liver and lung. No activity was observed in heart, kidney and spleen tissues and in the blood. Hexosamine production *in vitro* was found in liver [20, 21, 17], cornea [22] and colon tissues [27], as well as in connective tissue [6, 24] and in the human artery [11].

Some of the authors mentioned above have described experiments on tissues in which very slight or no hexosamine production was demonstrated. Considering that mucopolysaccharide production *in vivo* seems to be dependent upon the oxygen supply of the tissue, it was reasonable to suppose that similar effect can be obtained *in vitro* too, and even tissues showing no aerobic hexosamine production can be influenced to produce hexosamine under anaerobic conditions.

Material and method

Experiments were conducted on white rats weighing 180—220 g. The rats were killed by a blow on the nape and the tissues to be tested were excised and placed immediately in dishes kept in melting ice. The whole procedure was done as quickly as possible, since a loss of hexose phosphate transaminase activity was noted even at —20 °C [21]. The tissues were quickly minced with fine scissors, keeping the dish all the time in melting ice.

In determining hexosamine synthetase the method of POGELL and KOENIG [22] was used as modified by LELOIR and CARDINI [16]. An amount of 0.6—0.9 g tissue mince was placed in a Warburg flask, to which 0.5 ml of 1% glucose and 0.4 ml of 0.1% glutamine were added and the mixture was made up to a total volume of 3.2 ml by adding Krebs—Ringer bicarbonate buffer solution (pH 7.4). The flask connected with a manometer was shaken at a rate of 70/min. at 37 °C for 3 hours. For anaerobic measurements the tubes were flushed with a mixture of gas containing 95% N_2 and 5% CO_2 (traces of O_2 may have been present in the gas mixture). For aerobic measurements the flasks contained air. The parallel anaerobic and aerobic systems were shaken simultaneously.

At zero time controls and three hours later, the contents of Warburg flasks were washed quantitatively into glass tubes supplied with glass stoppers, then concentrated HCl was added until the final concentration of 4N was attained. After simultaneous hydrolization of all the parallel samples their hexosamine content was determined by Boas' method [4]

Results

The results of determinations are illustrated in Figs 1—5, which show the hexosamine production obtained in 3 hours at 37 °C under aerobic (light bars) and anaerobic (dark bars) conditions. Hexosamine content of fresh tissues at zero time was rather varying: 46.2—85.1 mg per 100 g liver, 57.7—119.0 mg per 100 g lung, 54.5—98.4 mg per 100 g skin, 98.2—143.8 mg per 100 g kidney and 82.7—103.0 mg per 100 g spleen. Therefore, for the sake of perspicuity,

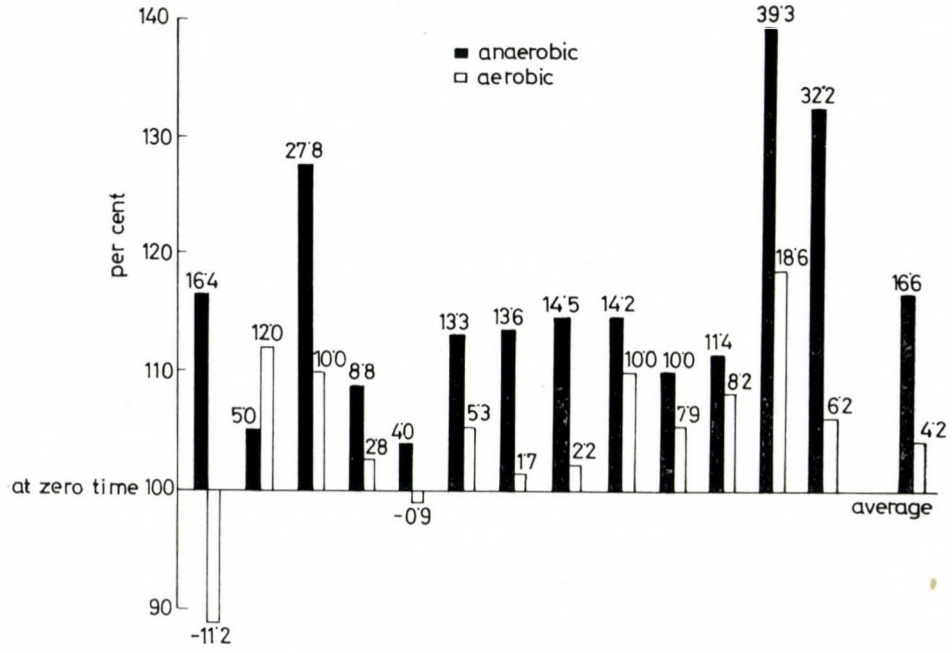

Fig. 1. Hexosamine production *in vitro* by minced rat liver during 3 hours at 37 °C expressed in per cent of hexosamine content at zero time

hexosamine content at zero time has been indicated as 100% and hexosamine production during 3 hours has been expressed in per cent of the zero-time value. The values expressed by the bars represent the averages of parallel measurements.

Fig. 1 shows that hexosamine was produced in the liver in all 13 cases. Higher amounts were measured under anaerobic conditions than under aerobic ones in 12 cases. The opposite was observed in one case. The mean value of anaerobic production was 16.6%, while the amount of hexosamine produced in aerobic environment was only 4.6%.

In the lung tissue (Fig. 2) hexosamine was produced in 11 cases, out of 15. Higher hexosamine production was obtained under anaerobic conditions in

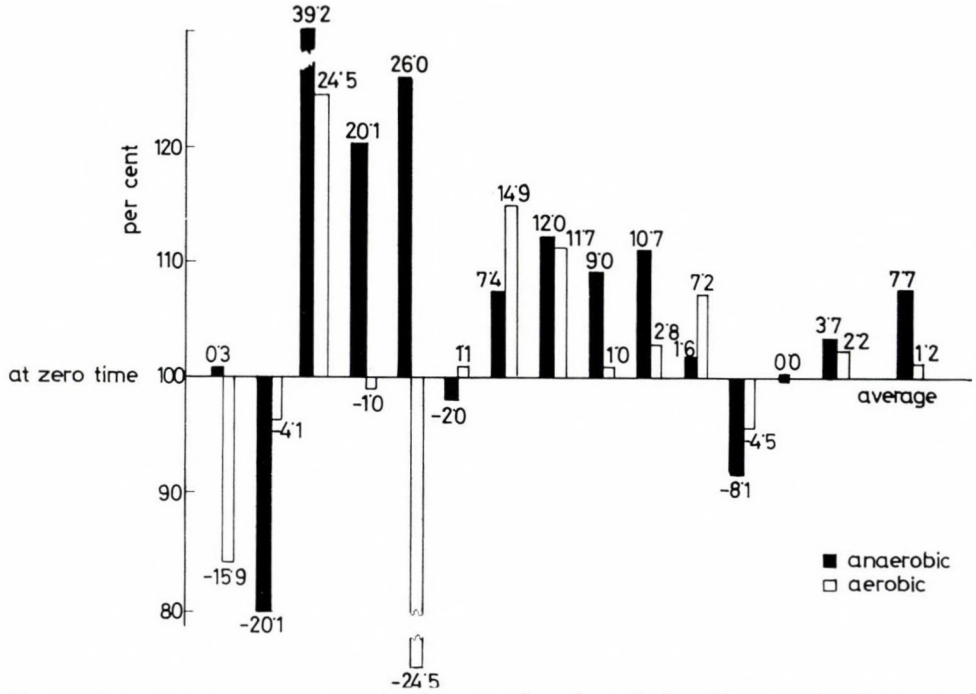

Fig. 2. Hexosamine production *in vitro* by minced rat lung during 3 hours at 37 °C expressed in per cent of hexosamine content at zero time

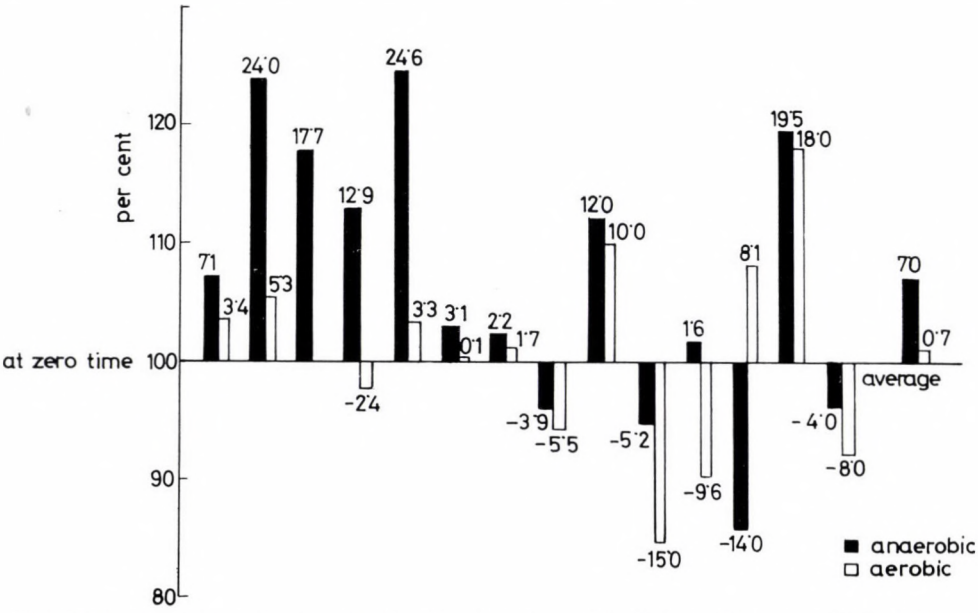

Fig. 3. Hexosamine production *in vitro* by minced rat skin during 3 hours at 37 °C expressed in per cent of hexosamine content at zero time

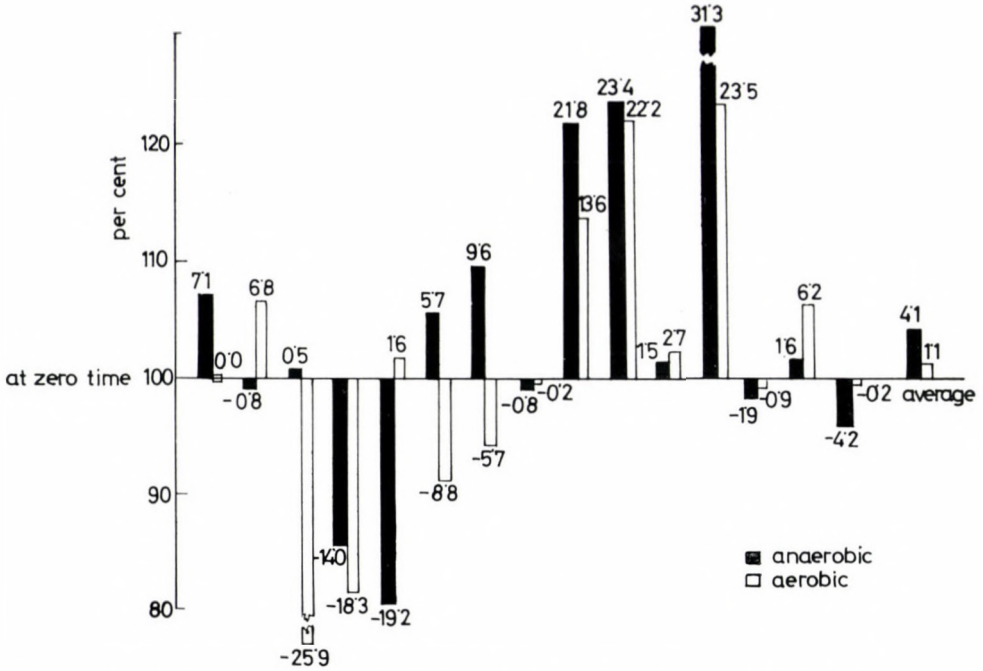

Fig. 4. Hexosamine production *in vitro* by minced rat kidney during 3 hours at 37 °C expressed in per cent of hexosamine content at zero time

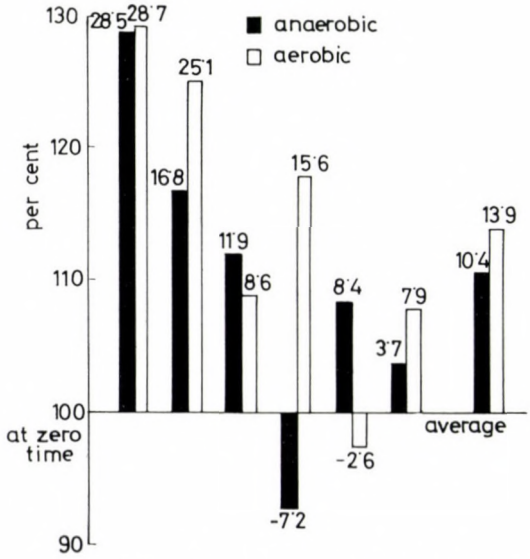

Fig. 5. Hexosamine production *in vitro* by minced rat spleen during 3 hours at 37 °C expressed in per cent of hexosamine content at zero time

8 out of 11 cases. On the average the hexosamine production was 7.7% (anaerobic) and 1.2% (aerobic).

Of 14 determinations in rat skin-tissue (Fig. 3) 11 showed hexosamine production. The production was more intensive in anaerobic milieu in 9 cases. The average production was 7.0% in anaerobic and 0.7% in aerobic milieu.

Of 15 measurements in rat kidney tissue (Fig. 4) hexosamine production was demonstrated in 11, of which the anaerobic production was higher in 7 cases. The average anaerobic production was 4.1%, the aerobic 1.1%.

The rat spleen (Fig. 5) behaved differently. Six determinations were made, which all yielded positive results and higher values were obtained under aerobic conditions in 4 cases. The mean hexosamine production was 10.4% in the presence of oxygen and 13.9 in its absence.

Determinations of cardiac and thigh muscles (5 measurements in each) failed to demonstrate hexosamine production both under aerobic and anaerobic conditions.

Discussion

Increased hexosamine production was noted in the liver, lung, skin, kidney and spleen tissues of the rat. The production was sometimes more than 30% of the original hexosamine content of the tissue. The averages calculated from the individual values — both in anaerobic and aerobic environment — do not, however, give an entirely reliable picture since, besides the high values obtained in the majority of the cases, in some samples no hexosamine production was demonstrable at all. This is by no means an evidence of the absence of hexosamine synthetase activity in that tissue; it may have resulted from some methodical error owing to the high sensitivity of the enzyme [21]. In order to obtain sufficient material for parallel measurements the removal of the tissue requires rather long time which may cause considerable loss of enzyme activity even if the material is kept at 0 °C, all the more that loss of activity of this enzyme may occur even at —20 °C. Consequently, the hexosamine content of the tissue may decrease owing to the activity of the more resistant glycolytic enzyme system which can decompose hexosamine. The average values comprising both positive (increased) and negative (decreased or nil) determinations do not seem to reflect the actual rate of hexosamine production. It seemed therefore reasonable to start from cases in which hexosamine production was evident.

Thus, hexosamine production was demonstrated in 100% of the liver and spleen samples, in 79% of the skin and kidney samples and in 73% of the lung samples. The hexosamine production was more intensive in anaerobic than in aerobic environment in 12 *vs.* 1 liver, 8 *vs.* 2 lung, 9 *vs.* 2 skin, 7 *vs.* 4 kidney and 2 *vs.* 4 spleen specimens.

It should be noted that owing to the high sensitivity of the enzyme, separation of morphologically different tissues within the same organ was not possible, though it would have been of importance, particularly in the spleen.

On the basis of the results obtained it may be concluded that minced tissues of rat liver, lung, skin, kidney and spleen are capable of producing hexosamine starting from glucose, in the presence of glutamine. The rate of hexosamine production was found to be dependent upon the oxygen supply of the tissue. For the hexosamine production by the liver, lung, skin and kidney tissues an anaerobic environment seems to be more favourable than an aerobic one, while for the spleen the experimental data do not suffice for a conclusion in this regard. Determinations on cardiac and skeletal muscles failed to yield positive results.

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HEXOSAMIN-SYNTHESE IN RATTENGEWEBEN BEI AEROBEN UND ANAEROBEN BEDINGUNGEN *IN VITRO*

Die Hexosamin-Produktion von Leber-, Lungen-, Haut-, Nieren-, Milz-, Herzmuskel- und Schenkelmuskelpräparaten wurde *in vitro* unter anaeroben (stark sauerstoffarmen) und aeroben Bedingungen parallel untersucht. Die Gewebe wurden nach der Methode von POGELL und KOENIG unter Zugabe von Glukose und Glutamin bei 37 °C drei Stunden inkubiert. Der Hexosamingehalt wurde mit dem Boas-Verfahren bestimmt. Leber, Lungen, Haut, Nieren und Milz zeigten in 73—100% der Messungen eine verschieden starke, doch registrierbare Hexosamin-Produktion. Im Muskelgewebe war kein Anstieg des Hexosamingehaltes aufzuzeigen. In den eine Hexosamin-Synthese aufweisenden Geweben hing das Ausmaß der Produktion von der Sauerstoffversorgung ab: die Leber wies unter anaeroben Bedingungen in 12 Fällen einen größeren Hexosaminanstieg auf, und nur in einem Fall war in der Leber die Synthese unter aeroben Bedingungen größer. Bei der Haut beträgt dieses Verhältnis 9 : 2, bei den Nieren 7 : 4, während die Lunge im Verhältnis 8 : 3 eine höhere anaerobe Synthese zeigte. Die Hexosaminsynthese der Milz wird durch aerobe Verhältnisse begünstigt: gegenüber zwei anaeroben Messungen wurden unter aeroben Bedingungen in 4 Fällen höhere Werte ermittelt.

ПРОИЗВОДСТВО ГЕКСОЗАМИНА ТКАНЯМИ КРЫСЫ IN VITRO В АЭРОБНЫХ И АНАЭРОБНЫХ УСЛОВИЯХ

Производство гексозамина препаратами печени, легких, кожи, почек, селезенки, миокарда и бедренной мышцы крыс изучалось *in vitro* в аэробных и анаэробных (очень слабое снабжение кислородом) условиях. Ткани были инкубированы по методу Погелл и Кениг в присутствии глюкозы и глютамина при температуре в 37° С на протяжении 3 часов. Измерение содержания гексозамина проводилось по методу Боаса. Печень, легкие, кожа, почки и селезенка в 73—100% случаев показали регистрируемое производство гексозамина в различной интенсивности. В мышечной ткани повышения содержания гексозамина не удалось выявить. В тканях, показывающих синтез гексозамина, интенсивность производства зависит от снабженности кислородом. Печень в 12 случаях показала более значительное повышение содержания гексозамина в анаэробных условиях, а лишь в одном случае в аэробных. В коже это соотношение 9 : 2, в почках оно 7 : 4, тогда как в легких в анаэробных условиях был выявлен более высокий синтез в соотношении 8 : 3. В селезенке синтезу гексозамина благоприятствуют аэробные условия: при 4 измерениях было выявлено более высокое производство, по сравнению с двумя измерениями, показывающими повышенный синтез в аэробных условиях.

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LIGHT MICROSCOPIC EXAMINATION OF THE NERVOUS SYSTEM OF TUNICATA*

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Synopsis

The central nervous system was examined in 12 ascidian and an appendicularian species histologically with special regard to neurosecretory phenomena. In the brain, besides neuroglia cells, four cell types were found: (1) unipolar giant cells situated on the surface of the outermost cortex; (2) small unipolar cells located among the giant cells or within a deeper layer; (3) bipolar cells mostly located at the border between the cortex and medulla; (4) multipolar cells in the so-called central gray matter. The fibres originating in the cells bend towards the two ends of the ganglion where they leave the ganglion in the form of nerves. Three types of Gomori-positive and three types of Gomori-negative intracellular substances are described. One of the Gomori-positive substances, the so-called fine-granulated substance in the small cells, shows great resemblance — as for its appearance, size and staining properties — to the neurosecretory substance known in other species. The neurosecretory activity of the brain may be attributed to the cells containing this type of granule. Another Gomori-positive substance, to be found in the giant cells, is rough-granulated, it is most probably a pigment; it shows a yellowish-brown colour in native preparations and gives an intensive staining with aldehyde-fuchsin. The third type of the Gomori-positive substance gives a pale reaction with aldehyde-fuchsin. This as well as the orangeophilic materials and those staining by light green cannot be identified exactly on the basis of the present investigations.

Introduction

Several works have dealt with the nervous system of Tunicata, with its function [2, 3, 5, 6, 7, 10, 11, 12] and structure [8, 9, 16, 20, 21, 22, 24, 25]. In 1952 Mazzi [26] described substances detectable in the nervous system of *Ciona intestinalis* with selective staining methods. Similar investigations were performed by Scharrer [29] in 1953 on *Molgula manhattensis*. Neurosecretory cells were described by Dawson and Hisaw in the nervous system of Tunicata first in 1956 [8] and then in 1964 [9].

Since the available light-microscopic data are in several regards contradictory and give no complete picture of the structure of the nervous system, it seemed to be justified to complete our knowledge on this subject with further morphological data.

The present study involving our results obtained by general histological and silver impregnation methods was made as an introduction to further comparative and ultra-structural investigations into the nervous system of various Tunicata.

*Supported by grant from the Anton and Reinhard Dohrn foundation.

Material and method

The tunicates used for material were collected from the Gulf of Naples throughout a year. Part of the collected specimens was fixed *in toto*, from others the separated neural complex only was fixed. A great part of the material was fixed in Bouin's fixative, embedded in methyl benzoate—paraffin and sectioned serially at 6 μ in cross and longitudinal planes. The materials fixed in Bouin's fluid were stained with Gabe's paraldehyde fuchsin, Gomori's chrome hematoxylin and — after having been oxidized with performic acid — with alcian blue. The PAS reaction was performed with and without digestion. For other fixatives, besides the Bouin's fluid, Carnoy's and Helly's fluids, the Susa fixative after Heidenhain and Ca formalin were used. The materials fixed in the latter fixatives were stained with the following methods: Heidenhain's iron haematoxylin, Mallory's procedure, Azan method and May—Grünwald—Giemsa staining. Also Bodian's impregnation method was applied on a part of the material [28].

List of the used Tunicata species

Oicopleure dioica (Appendiculariae)	36	specimens
Clavellina lepadiformis	32	,,
Rhopalea neapolitana	30	,,
Ascidiella aspersa	42	,,
Ascidiella scabra	25	,,
Ascidia malaca	25	,,
Ascidia mentula (MÜLLER)	82	,,
Ascidia oblonga	4	,,
Phallusia mammillata	40	,,
Phallusia fumigata	15	,,
Microcosmus sulcatus	12	,,
Halocynthia papillosa	10	,,
Ciona intestinalis	120	,,

Results

The brain of tunicates is represented by an ellipsoidal ganglion in the medio-dorsal line between the oral and atrial siphons. It is embedded in the loose connective tissue located between the ectoderm and the pharynx. Nerves arise from the ganglion orally and atrially.

The ganglion is enclosed in a thin connective tissue layer which penetrates deeply the central fibrous matter among the ganglion cells. The loose connective tissue surrounding the ganglion is rich in blood sinuses, but no capillaries were found within the ganglion itself. A cortical zone built up of cells is immediately beneath the connective tissue capsule, where it forms one layer or more. Under this, a network of fibres extending from the cells forms the medullar zone. In the centre of the medullar zone small nerve cells and glia cells can be found which constitute the central gray matter (Figs 1 and 2).

Nerve cells

Cells of the cortical zone. Immediately beneath the connective tissue capsule, peripherally large ganglion cells or giant cells, are to be found. Their average diameter is 15—20 μ, in *Phallusia mammillata* they can reach 30—35 μ in diameter. The giant cells are unipolar, and pyriform in shape. Their thick neurite passes through the cortical zone and enters the fibrous matter (Fig. 3).

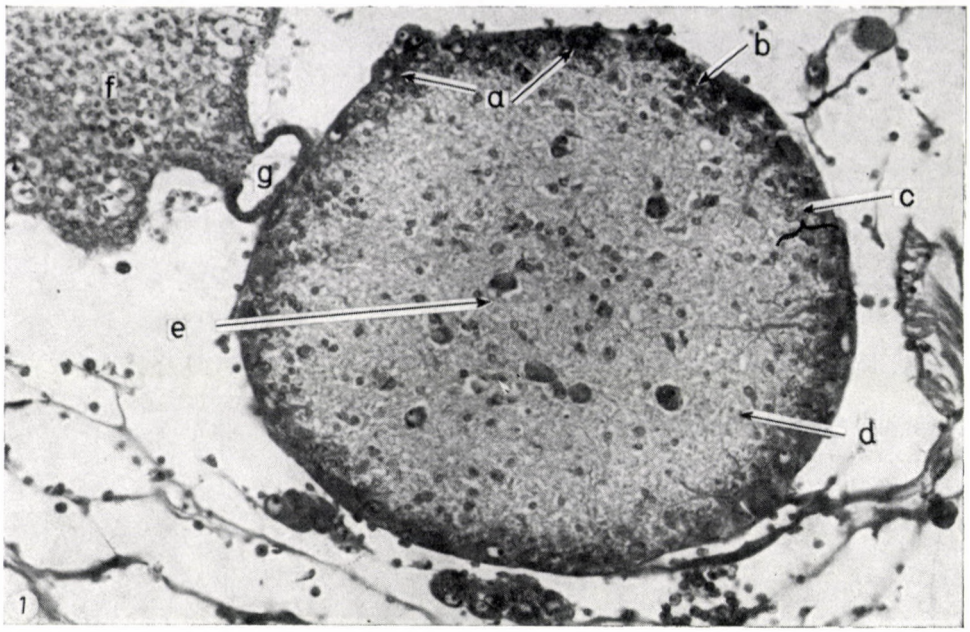

Fig. 1. Cross-section from the ganglion of *Ciona intestinalis: a)* giant cells, *b)* small cells, *c)* cortical zone, *d)* medullar zone, *e)* central gray matter, *f)* duct of the neural gland. May, Grünwald and Giemsa stain

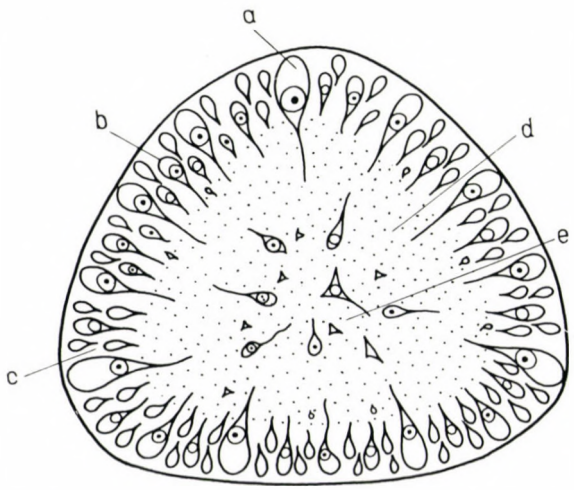

Fig. 2. Cross-section of the ganglion of *Ciona intestinalis* (schematic drawing): *a)* giant cells, *b)* small cells, *c)* cortical zone, *d)* medullar zone, *e)* central gray matter

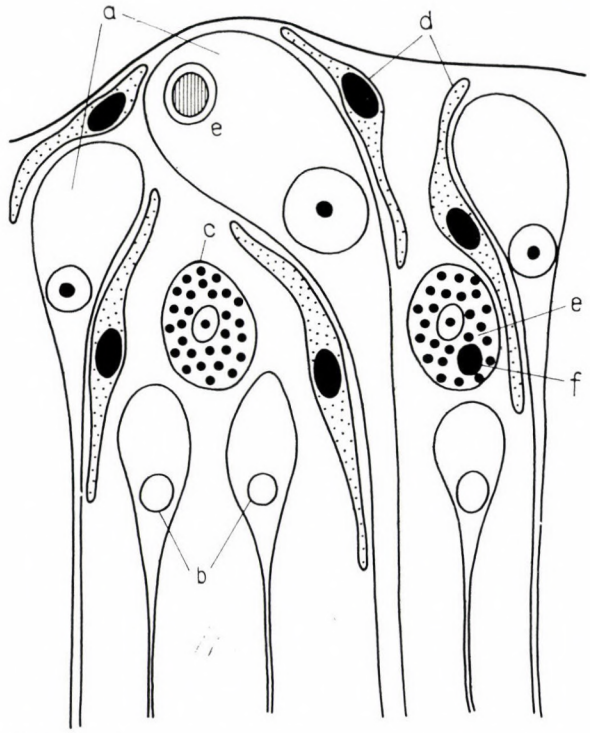

Fig. 3. Cortical zone of the ganglion (schematic drawing): *a)* giant cells, *b)* small cells, *c)* Gomori: positive secretion cells, *d)* glia-cells, *e)* light green-positive granule, *f)* orangeophilic granule

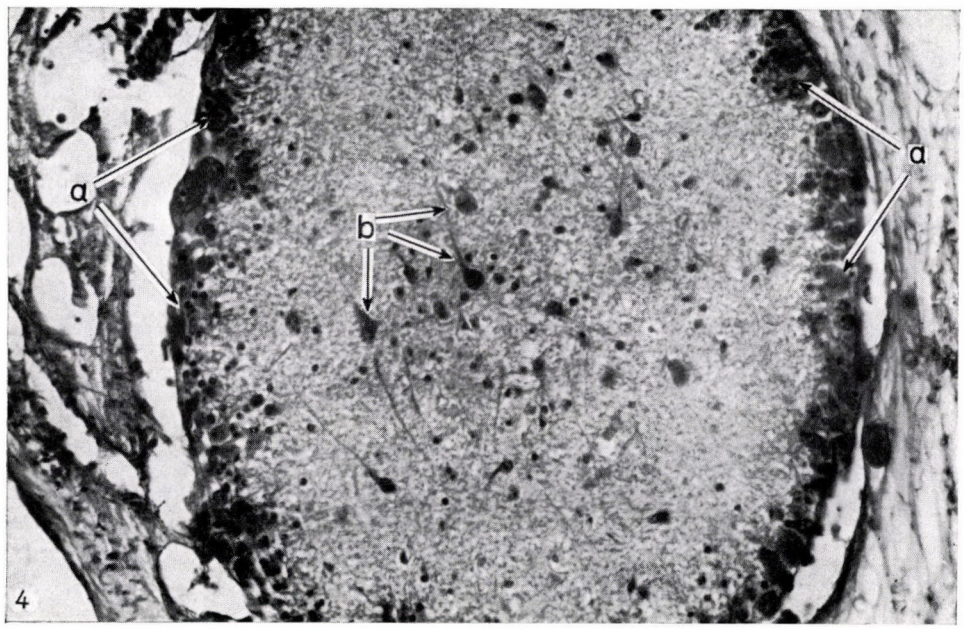

Fig. 4. Cross-section from the ganglion of *Phallusia mammillata: a)* cortical zone, *b)* cells of the central gray matter. May, Grünwald and Giemsa stain

Among and beneath the giant cells, small cells 8—10 μ in diameter are located in 2 or 3 layers. These cells are unipolar and have a spherical or, in many cases, a pear-shaped form.

Cells of the central gray matter. The central gray matter is built up of cells of various types. Among the cells numerous unipolar cells and fewer multipolar cells can be found. The process of the cells extends towards the cortical matter (Figs 1, 2, 4).

Elective staining substances in the nerve cells. (a) Numerous cells contain, among larger ones, Gomori-positive granules about $\frac{1}{4}$ μ in diameter. The fine granules stain bluish-violet with aldehydefuchsin and bluish-green with azan. Previous oxidation with permanganate — sulphuric acid intensifies their azan-staining. Their elective demonstration is possible with performic acid — alcian blue. As for their appearance, these granules can be compared with the neurosecretory substances. This type of granulation can be found within the unipolar small cells (Fig. 5). The perikaryon is sometimes filled up with this material which, like Herring-bodies appears, in many cases, also in the processes. Fine-granulated cells, three, four, or more in a group, occur in the whole cortical zone of the ganglion (Fig. 6). The cells containing this substance have been found in all species examined by us. It is remarkable that in *Oicopleura dioica* (Appendicularia), the brain of which shows great structural differences compared to that of ascidians, similar, fine-granulated, Gomori-positive substance was observed in nerve cells of the so-called forebrain and middle-brain. Similar granulation was found in nerve cells of the tail ganglion (Figs 7 to 10).

In the ascidians, besides the fine-granulated substance, a rough-granulated, Gomori-positive material is observable the granules of which are several times larger than those of the fine-granulated one. One type of these stains intensive blue with aldehyde-fuchsin. The conglomerated granules can form configurations similar to roll of coins or that of other types. Another type of the rough-granulated substance constitutes large, homogeneous inclusions staining pale red with aldehyde-fuchsin. Among the species examined by us only *Phallusia mammillata* has both types of the rough-granulated, Gomori-positive substance in its giant cells (Fig. 11).

(b) The Gomori-negative material to be found in the cells can be classified into 3 types: one stains with orange-G, another with light green and the third with aniline blue.

Inclusions stained by orange-G can generally be found in the cells containing the fine-granulated Gomori-positive substance. Their first appearance corresponds to the nucleolus both in size and staining. In a single cell 2 or 3 inclusions may occur (Fig. 12). These granules, after fusing, form smaller or larger hyalin-like bodies which displace the nucleus to one pole of the cell

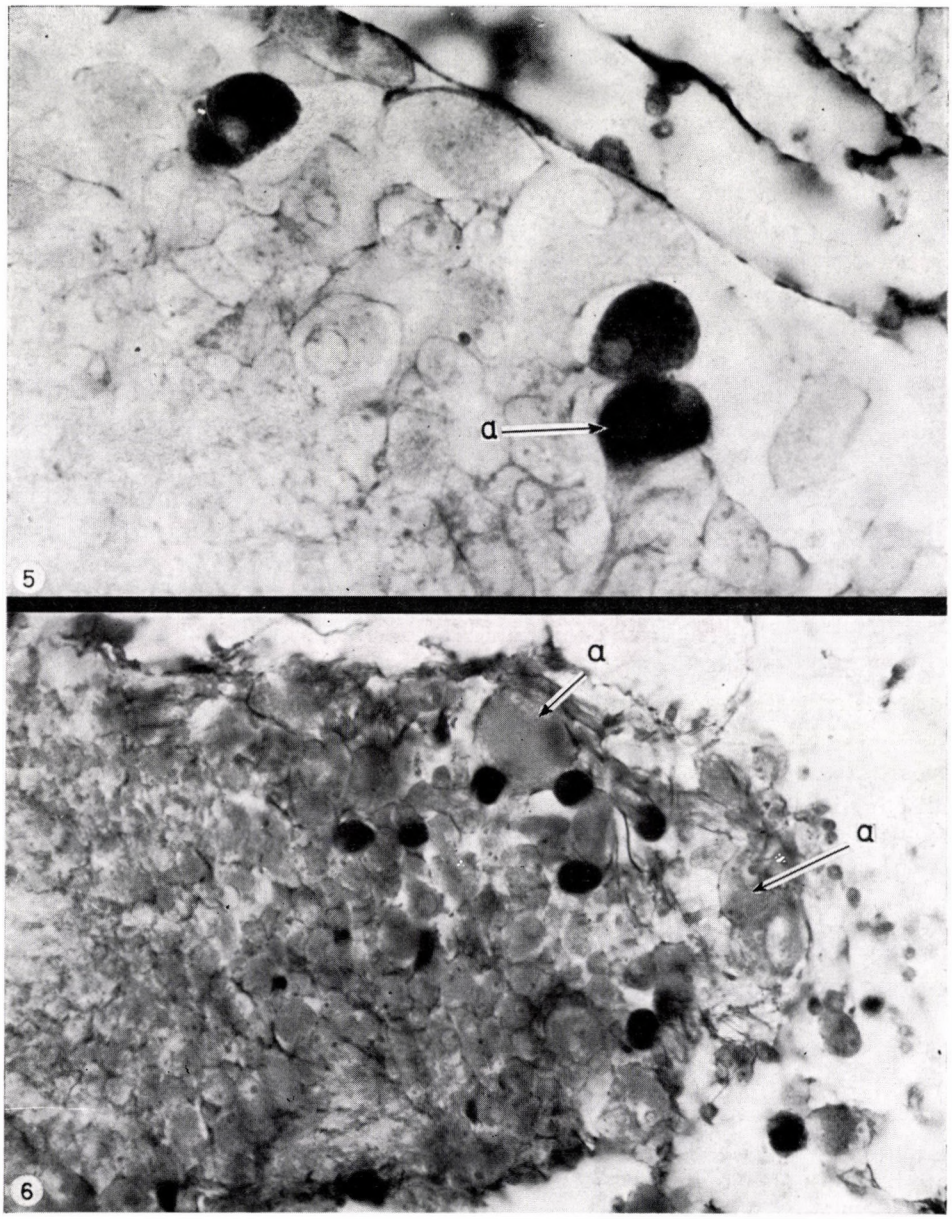

Fig. 5. Cells containing Gomori-positive fine granulation in the ganglion of *Ascidia mentual: a)* orangeophilic substance among the granules. Aldehyde-fuchsin stain

Fig. 6. Cross-section from the ganglion of *Ascidia mentula.* The cells containing Gomori-positive fine granules are arranged in groups. Aldehyde-fuchsin stain

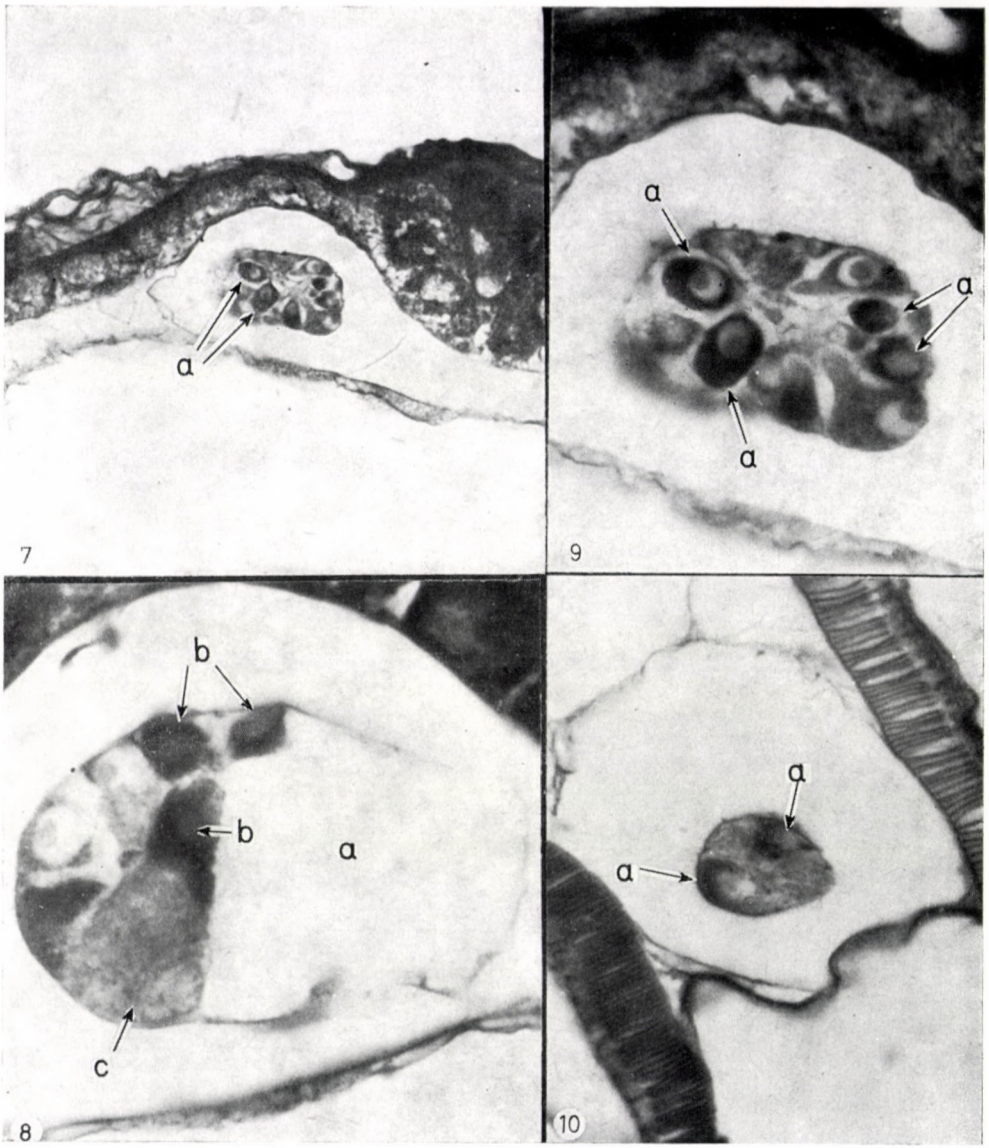

Fig. 7. Cross-section from the fore-brain of *Oikopleura dioica: a)* cells containing Gomori-positive granules. Aldehyde-fuchsin stain

Fig. 8. Cross-section from the middle-brain of *Oikopleura dioica: a)* sensory vesicle, *b)* Gomori-positive cells, *c)* cell of the hind-brain. Aldehyde-fuchsin stain

Fig. 9. Cross-section from the fore-brain of *Oikopleura dioica: a)* Gomori-positive cells. Aldehyde-fuchsin stain

Fig. 10. Cross-section from the tail-ganglion of *Oikopleura dioica: a)* Gomori-positive cells. Aldehyde-fuchsin stain

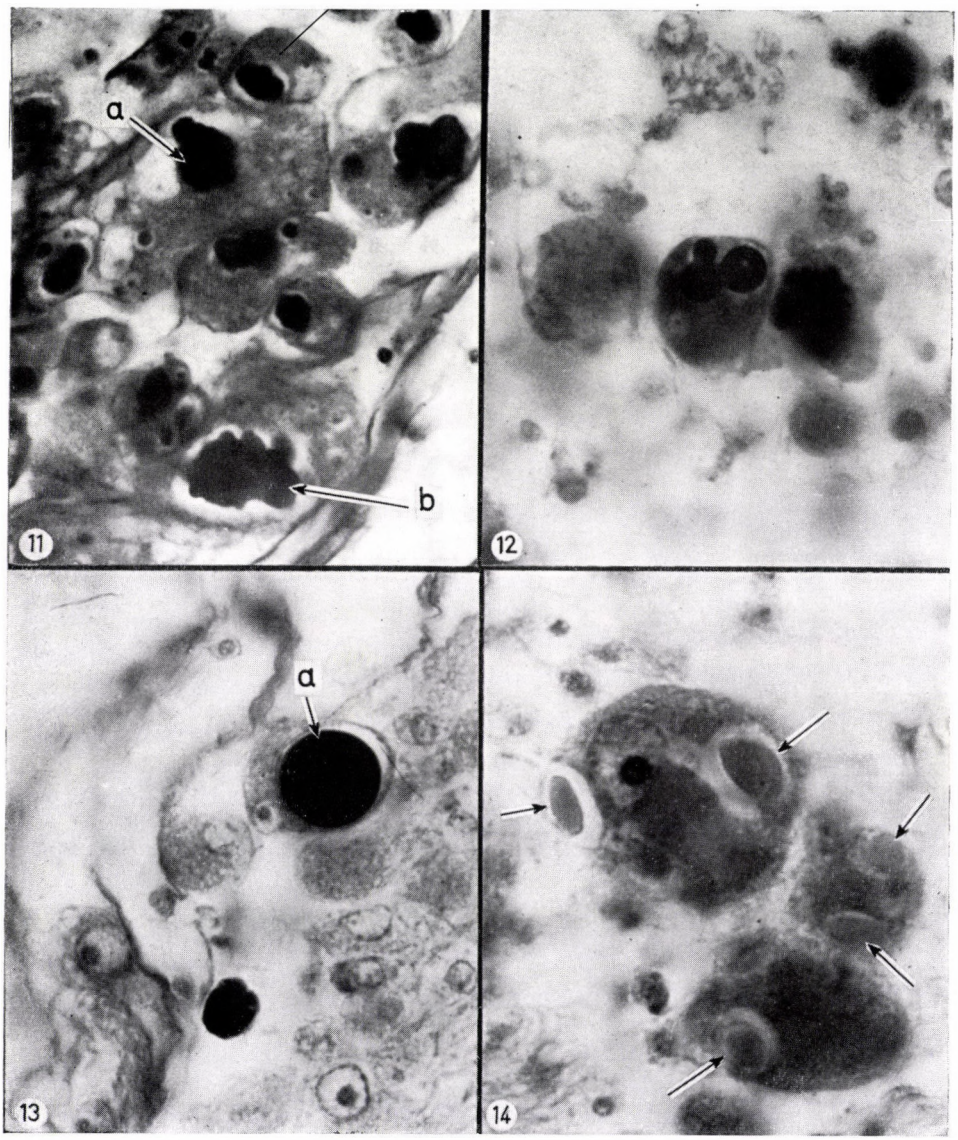

Fig. 11. The ganglion of *Phallusia mammillata: a)* rough-granulated, Gomori-positive substance staining intensively, *b)* rough-granulated Gomori-positive material of a pale staining. Aldehyde-fuchsin stain

Fig. 12. Ganglion of *Ascidia mentula:* orangeophilic granules in the small cells. Aldehyde-fuchsin stain

Fig. 13. Ganglion of *Ascidia mentula: a)* small cell totally filled up with orangeophilic substance. Aldehyde-fuchsin stain

Fig. 14. Ganglion of *Ascidia mentula:* light green-positive material in a giant cell (arrows). Aldehyde-fuchsin stain

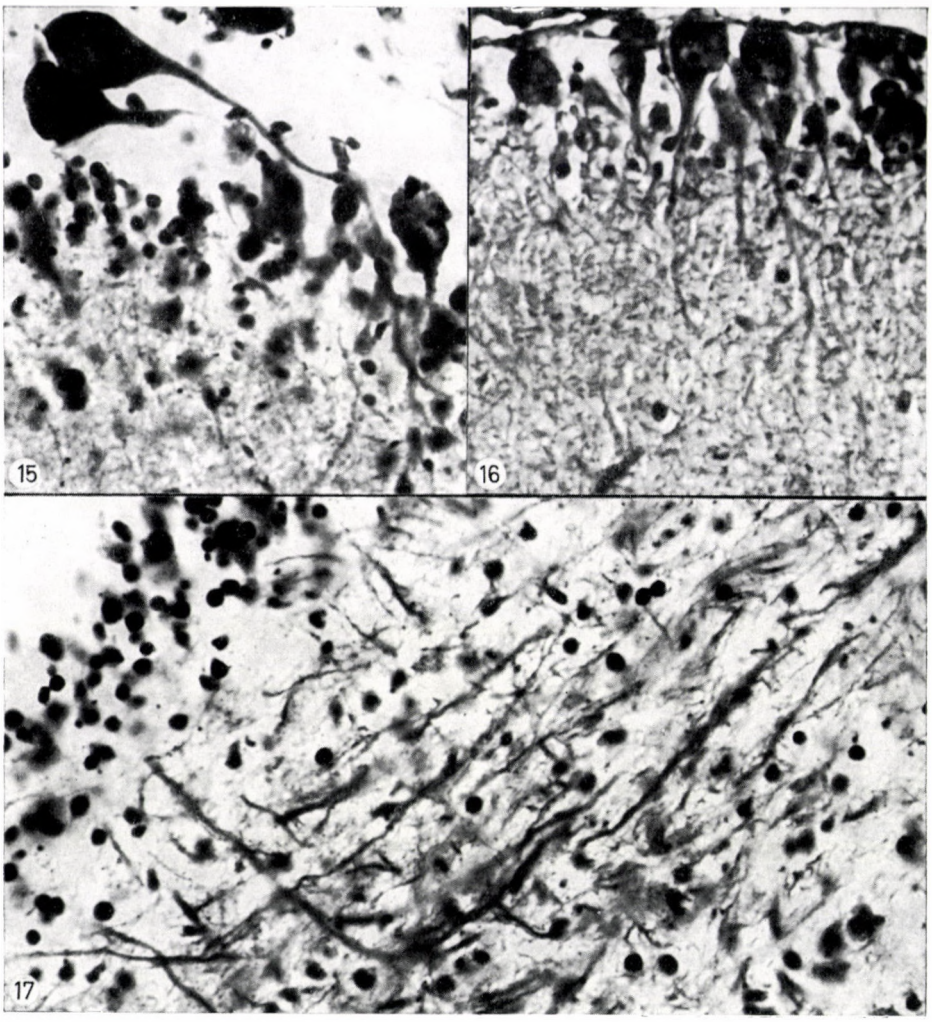

Fig. 15. Ganglion of *Ascidia scabra:* giant cells with fibres travelling through the cortical zone towards the medullar zone. Bodian impregnation

Fig. 16. Cross section from the ganglion of *Ascisiella aspersa:* giant and small cells. Bodian impregnation

Fig. 17. Cross section from the ganglion of *Ascidia mentula.* Fibres bending at right angle in the medullar zone. Bodian impregnation

(Fig. 13). Parallel with the growth in size of the granules the fine-granulated, Gomori-positive material disappears from the cell.

The granules staining with light green appear in general in cells containing no Gomori-positive material. Among the species studied by us they can be found in giant cells of *Ascidia mentula* in considerable quantity. In form of the first appearance the granules stained with light green are as large as a nucleolus

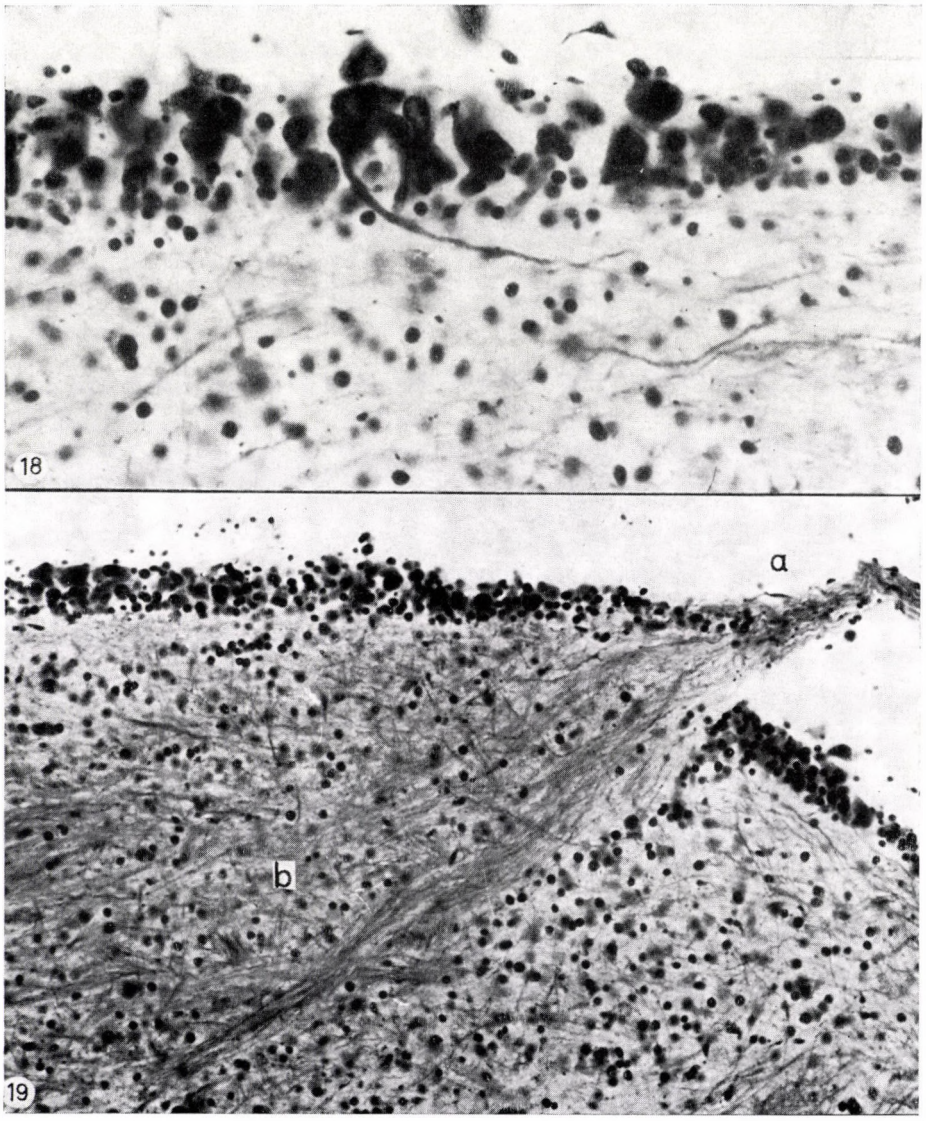

Fig. 18. Longitudinal section from the ganglion of *Ascidia mentula:* unipolar cells with a neurite running parallel with the longitudinal axis of the ganglion immediately beneath the cortical matter. Bodian impregnation

Fig. 19. Longitudinal section from the ganglion of *Ascidia mentula: a)* nerve fibres arising from the oral end of the ganglion, *b)* crossing of fibres travelling from the opposite sides. Bodian impregnation

and are always surrounded by a narrow, light area. Two or three are seen in a single cell. These granules either fuse and form larger drop-like bodies or, by growing further, constitute several isolated "pools" within the cytoplasm. The "pools" contain a homogeneous material (Fig. 14).

In giant cells of *Ciona intestinalis* a substance of similar appearance has been found. This, however, does not stain with light green. In freshly stained preparation it shows a blue colour. It is also located in a vacuole, which is, however, totally filled by its flocculated material.

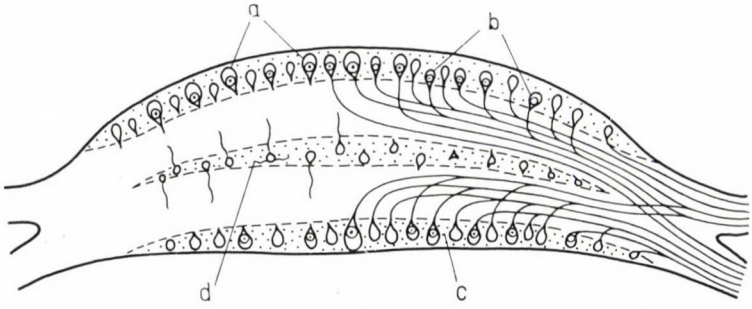

Fig. 20. Longitudinal section from the ganglion of *Ascidia mentula* (schematic drawing): *a)* large cells, *b)* small cells, *c)* cortical zone, *d)* central gray matter

Glia-cells

In the head ganglion glia-cells occur in relatively great number both among the nerve cells and in the fibrous matter. They are characterized by an elongated nucleus of compact structure. The glia-cells among the nerve cells are bipolar, while those within the fibrous matter are multipolar (Fig. 3). Both cell types contain glycogen. The multi-processed glia-cells form a well-defined glia-zone at the border between the cellular and fibrous matter. Their cytoplasm is rich in glycogen granules.

Fibrous matter of the ganglion

In the medullar zone of the ganglion fibres arise from the cells of the cortical substance as well as from those of the central gray matter (Figs 1, 4, 15). In cross sections of the ganglion fibres run radially from the cortical substance, towards the centre of the ganglion (Fig. 16). As it is observable in longitudinal sections, the fibres travelling inwards bend in different layers of the medullar matter and run further along the longitudinal axis of the ganglion towards its two ends (Figs 17, 18). Part of the fibres run on the same side, while others cross to the opposite side (Figs 19, 20). The fibres run parallel only within the ganglion. In the species *(Ascidia mentula, Phallusia mammillata)*, where there are also nerves arising from the ganglion laterally, the nerve pathways can be seen also inside the ganglion.

Discussion

According to earlier authors, the nervous system of tunicates representing forerunners of the vertebrates consists of a central fibrous matter and a peripheral cell layer. The cortical part is variable in thickness. According to LORLEBERG [22], in *Perophora listeri* the ganglion cells are arranged in 3 or 4 layers at the ventral side of the brain, on the site where the ciliary groove adjoins the canal, whilst on the dorsal side they form a single layer. In *Dendrodoa grossularia*, VAN BENEDEN [cit. in 20] made similar observations. According to LORLEBERG [22] in colony-forming ascidians the cortical ganglion cells are small, 2 to 5 μ on the average; in *Perophora*, giant cells even up to 13 μ in diameter occur among the small cells.

According to HILTON [16], the ganglion cells are unipolar except those situated in the central fibrous matter, which he considers multipolar. He described the large and middle-large cells situated cortically as motor cells, the smaller, multipolar ones living deeper partly as connective.

In the opinion of LORLEBERG [22], the large cells located peripherally represent connective tissue cells migrated in.

The nerves are most often concentrated at the anteroposterior line of the ganglion and show individual differences in ramifying when arising from the ganglion. In the simple ascidians, all the larger nerves arise from the ganglion anteriorly and posteriorly, forming double trunks. Two strong trunks leave the ganglion at the oral end, two at the aboral end, the roots forming later further ramifications [20]. There are species (e.g. *Ascidia mentula)* where instead of the double main trunks, several small fibre-boundles arise from the two ends of the ganglion; there are even nerves which arise laterally.

Our observations made on the general structure and the cellular and fibrous substance of the ganglion partly confirm, partly modify or complete the data of earlier authors. In the ascidian species examined by us recently, we have established that the structural species-differences manifest themselves mainly in the number of cells constituting the cortical substance and in the size of cells, in the distribution of the nerves arising from the two ends of the ganglion and, finally, in the presence or absence of nerves arising laterally. Differences can be found, in addition, in the developmental stage of the central gray matter. This is most differentiated in *Phallusia mammillata*, less developed in the colony-forming ascidians.

We cannot support LORLEBERG's observation that the peripheral large cells would represent connective tissue cells. Though in impregnated sections we could not find dendrites of these cells, their structure is the same as that of the nerve cells; their thick neurite entering the ganglion can well be observed. We observed no similar cells in regions located farther from the ganglion.

We cannot confirm either the data of earlier authors [20, 22] suggesting that in the ganglion, at the site neighbouring the neural gland, the cortical zone is

thicker than on the opposite side. In contrast to this, we have found that in the neighbourhood of the neural gland the small cells and the glia cells are chiefly predominating, whilst, on the opposite side, large cells are in greater number.

Earlier authors, though used various staining methods for studying the nervous system of tunicates, did not observe any phenomenon suggestive of the secretory character of the nerve cells. HILTON [16] alone has observed, in nerve cells of *Ascidia atra (Tunica nigra)*, paranuclear vacuoles which were filled, from time to time, with pigment. Later on, MAZZI [26] and SCHARRER [29] have studied more thoroughly the nervous system of tunicates, but could not observe any neurosecretory phenomenon.

DAWSON and HISAW [8] briefly reported on the occurrence of neurosecretory cells in the ganglion of tunicates and, later, completed their studies with observations made on several specimens of three species.

In our own investigations we have found phenomena suggestive of neurosecretion in each of the species studied. In different neurons we could demonstrate several Gomori-positive and negative inclusions. In our opinion the fine-granulated Gomori-positive material may be regarded as a neurosecretory material in the classical sense. This is shown, besides staining properties, by the equal size of the granules and their dimension (about $^1/_4$ μ) which corresponds to the elementary granules of neurosecretion described in the literature. The material can fill up the perikaryon and may be observable even in the neurite, where it takes on a form similar to the Herring-bodies.

This fine-granulated substance is mainly found in the so-called small cells; we suppose, for this reason, that in the tunicates one type of these cells represents the neurosecretory cells. The variable quantity of granules and the vacuoles may represent different stages of a secretory cycle. Granulated processes of these cells disappear, after shorter or longer pathways, in the fibrous matter. In nerves arising from the ganglion such fibres could not be observed. This does not mean that these fibres never leave the ganglion, for the neurites may contain granules in quantities not demonstrable by light microscopy. The fact that in the animals studied by us most cells showed not an active, but a so-called "storing" form, supports this possibility. If, however, the fibres terminate in the fibrous matter itself, the secretion can be discharged into the wide interstitial space among fibres and cells.

In the large nerve cells of *Phallusia mammillata*, the substance staining ntensive blue with aldehyde-fuchsin and appearing in granules of variable form and size, shows in native preparation a yellowish-brown colour; therefore it is most probably a pigment, possibly lipofuscin.

The same cells may contain large quantities of another substance which stains pale red seems to be homogeneous. This substance often fills the cytoplasm totally and resembles the bodies described by DAWSON and HISAW [9] in

the ganglion of *Chelyosoma productum*. The true nature of this substance cannot be clarified on the basis of our present investigations.

The significance of the homogeneous granules of irregular shape and variable size, contained by the large cells on the surface of the ganglion and staining well with light green has also remained unknown. They seem not to represent a secretory process. Nevertheless, the permanent presence of cells containing this material, the strikingly loose chromatin-structure of the nucleus as well as the enlarged nucleolus are suggestive of a hitherto unknown phenomenon within the nerve cells.

An electron microscopical study of the present material is in progress.

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LICHTMIKROSKOPISCHE UNTERSUCHUNGEN AM NERVENSYSTEM VON TUNIKATEN

Das Zentralnervensystem von 12 Ascidien und einer Appendicularienart wurde mit histologischen Methoden unter besonderer Berücksichtigung neurosekretorischer Phänomene untersucht. Es wurde festgestellt, daß im Cerebralganglio n außer den Gliazellen vier Typen von Nervenzellen vorhanden sind. (1) Riesenzellen, die unipolar sind und sich an der Oberfläche der äußersten Rindenzone anordnen. (2) Kleine Zellen, die ebenfalls unipolar und unmittelbar zwischen den großen Zellen oder in der tieferen Schicht lokalisiert sind. (3) Bipolare Zellen, die meist an der Grenze von Rinde und Mark zu finden sind. (4) Multipolare Zellen, die in der sogenannten zentralen, grauen Substanz liegen. Die aus den Zellen austretenden Fortsätze nehmen nach einer längeren-kürzeren Strecke einen bogenförmigen Verlauf auf und ziehen zu den beiden Enden des Ganglions zur Bildung der dort austretenden Nerven. In den Zellen wurden drei verschiedene Gomori-positive und drei Gomori-negative Stoffe beobachtet. Unter den Gomori-positiven Substanzen zeigt das in den kleinen Nervenzellen auftretende, sogenannte feingranuläre Material in bezug auf seine Form, Größe und Färbbarkeit große Ähnlichkeit mit dem in anderen Tierarten bekannten neurosekretorischen Material. Es wird angenommen, daß die Zellen, die diese Granulaart enthalten, das neurosekretorische Phänomen im Cerebralganglion verkörpern. Der eine Typ des in den Riesenzellen auffindbaren, grobgranulären Materials, der sich mit Aldehydfuchsin intensiv anfärbt, zeigt im nativen Präparat eine gelbbraune Färbung; aller Wahrscheinlichkeit nach handelt es sich um ein Pigment. Die genaue Natur der dritten Gomori-positiven Substanz, die sich mit Aldehydfuchsin schwach anfärbt, ferner des mit Lichtgrün nachweisbaren Materials und der orangophilen Substanzen ist auf Grund gegenwärtiger Untersuchungen nicht feststellbar.

ИССЛЕДОВАНИЕ ЦЕНТРАЛЬНОЙ НЕРВНОЙ СИСТЕМЫ ОБОЛОЧНИКОВ В СВЕТОВОМ МИКРОСКОПЕ

Центральная нервная система 12 видов **Ascidiae** и одного вида **Appendiculariae** была изучена гистологическими методами исследования с особым вниманием на нейросекреторные явления. В мозге, помимо клеток нейроглии, присутствуют еще 4 типа клеток: (1) гигантские, униполярные клетки, располагающиеся на поверхности самой наружной коры; (2) мелкие, также униполярные клетки, помещающиеся непосредственно между гигантскими клетками или в более глубоком слое; (3) биполярные клетки, обнаруживаемые чаще всего на границе коркового и мозгового вещества; (4) мультиполярные клетки, находящиеся в так наз. центральном сером веществе. Волокна, исходящие из клеток, после более или менее длительного пути, лугообразно загибаются и идут к двум концам нервного узла, образуя отходящие там нервы. В клетках авторы описывают три типа Гомори-положительных и три типа Гомори-отрицательных веществ. Среди Гомори-положительных веществ присутствующее в мелких клетках так называемое тонкозернистое вещество, по своей форме, величине и окрашиванию показывает значительное

сходство с нейросекреторным веществом, известным у других видов. Полагается, что клетки, содержащие такое зернистое вещество, представляют собой нейросекреторные явления в мозге. Один тип выявляемого в гигантских клетках грубозернистого Гомори-положительного вещества, интенсивно окрашивающегося альдегидфуксином и показывающего в нативном препарате желтоватый оттенок, по всей вероятности относится к пигментам. Точную природу третьего Гомори-положительного вещества, слабо окрашивающегося альдегидфуксином, а также природу окрашивающегося светлозеленым (**Lichtgrün**) и оранжефильного веществ — на основе настоящих исследований еще нельзя установить.

Béla Aros, Budapest IX., Tűzoltó u 58, Hungary.

RECENSIONES

Biogenesis of natural compounds

Second edition. Ed. P. Bernfeld, Pergamon Press, Oxford (1967), pp. 1209, £ 17, 10 s.

The volume contains 19 chapters contributed by 24 authors including the editor. The exhaustiveness and thoroughness of the work is well illustrated by the fact that the author index covers 60, and the subject index 70 pages. The chapters written by scientists who have valuable contributions to the experimental development of the subjects discussed by them. All chapters deal with biosynthesis and biogenesis in respect of the following substances: amino acids (MATTOON); nucleotides (GUARINO); lipids (STRICKLAND); steroids (STAPLE); blood and bile pigments (BOGORAD and TROXLER); carbohydrates (BERNFELD); proteins (PETERS); nucleic acids (MANTSAVINOS and ZAMENHOF); conjugation and detoxication products (WILLIAMS); carotenoids and vitamin A (CHICHESTER and NAKAYAMA); watersoluble vitamins (CHELDELIN and BAICH); phenolic plant products (GEISMANN); tannins (HUMPHRIES); plant terpenes (NICHOLAS); lignins (NORD and SCHUBERT); rubber (BONNER); alkaloids (LEETE); fungal metabolites (WHALLEY); carcinogenic hydrocarbons (BOGER).

Most chapters — tending towards possible completeness — present a historical survey of the pertinent investigations, but only to that extent which is necessary for the understanding of current research work. The material covered by each chapter is well-proportioned, and reflects the recent knowledge on biosynthesis. Interrelations are clear by instructive tables and serial formulae indicating the hitherto explored paths of synthesis. The extent of the chapters is strictly adapted to the requirements of the subjects discussed; the chapter written by the editor on carbohydrates is exceptional in this respect: it covers 160 pages approaching thus in itself the size of a separate book. This does, however, by no means impair but rather raises the value of the volume. Each chapter is provided with an abundant and up-to-date bibliography. The book can be recommended first of all to chemists and biochemists, and also general biologists, zoologists, botanists, physicians, veterinarians and researchers working in related branches of science.

G. CSABA (Budapest)

Cell differentiation

A Ciba Foundation Symposium Ed. A. V. S. DE REUCK and J. KNIGHT, Churchill, London (1967), pp. 257.

In order to enrich our knowledge concerning the genetic role of nucleic acids and to be able to elucidate the regulation of protein synthesis it is necessary to modernize the methods of research, the concepts and aspects in the sphere of embryology. The symposium was arranged with these objectives in view. The most prominent researchers of cell differentiation contributed lectures to and discussed the pertinent problems. After an introductory address of ABERCROMBIE on terminology, the following subjects were discussed: the properties of the individual cell and the development of membranes (ROSENBERG); biochemical aspects of fertilization (BRACHET); nuclear transplantation and cell differentiation (GURDON); feed-back regulation and transmission of information (FELDMAN and BLEIBERG; AMBROSE; GEORGIEV); induction, inductors and synthetic activity (YAMADA; GROBSTEIN; BELL and MACKINTOSH); ribosomal genes (BIRNSTIEL); differentiation of tumours (PAUL; ET. and EM. WOLFF; WHISSON). The symposium was opened and closed by HADDOW.

The discussions printed out the numerous unsolved problems as regards application of the known facts and theories of genic activity to the cells of higher organisms. It is in any case evident that, as regards the developmental mechanics "classic" notions have given place to a completely new trend of research. Besides presenting exact experimental results, the material of the symposium raises new ideas and imparts new impulses for further investigations.

Excellent topography, instructive illustrations and a useful index mean great facilities for the reader.

G. CSABA (Budapest)

TROSHIN, A. S.: **Problems of cell permeability**

International Series of Monographs in Pure and Applied Biology. Modern Trends in Physiological Sciences Division. Vol. 26. Revised and supplemented edition.
Pergamon Press, Oxford (1966). Pp. XIV + 549, with 129 figures and 104 tables. £ 6.

The first edition of this work was published in 1956 in Russian, and its German translation appeared in 1958. The book here reviewed is a thoroughly revised and amplified English version of TROSHIN's work. The first three of the 12 chapters deal with the physical and chemical properties of cells as seen from the angle of their permeability. Chapters IV to VIII are devoted to problems concerning the penetration of non-electrolytes, organic acids, vital dyes and ions. Chapters IX and X discuss the mechanism of ion transport and the correlations between cellular permeability and metabolism. The two concluding chapters treat of the bioelectric properties of cells, as also of the protective action of nonelectrolytes against the structurally damaging effects of salt solutions.

Problems regarding the mechanism of cellular permeability constitute the central subject of TROSHIN's book. The whole work is, as a matter of fact, a polemic in which the author tries to defend his "sorption theory" against the champions of the "membrane theory" Although researches concerning biological transport mechanism have in many a respect passed beyond the material of the book, the arguments of TROSHIN are still instructive because they prevent the reader from forming a partial view and also because the author's reasonings and the raising of problems are good incentives for further experiments.

The work is especially valuable on account of the vast bibliographical list which, containing references to approximately 2,500 publications, presents the first overall survey of works dealing with the subject in the Russian, English, German and French languages.

G. GÁRDOS (Budapest)

HARRIS, H.: **Nucleus and cytoplasm**
Clarendon Press, Oxford (1968), pp. 142, 30s

Relying partly on his own experimental results, the author discusses the interactions between nucleus and cytoplasm. The work contains the following chapters: (1) The expression of genetic information; (2) The genetic information model; (3) The search for the messenger; (4) Regulation; (5) Hybrid cells; (6) Differentiation.

While it is now perfectly clear that protein synthesis is determined by instructions coded in the sequences of DNA, finer details and mechanism of the process are still rather obscure. Questions regarding conditions of time, spatial arrangement and quantity emerge in various forms in connection with the problems of transcription and translation. It is known that the continued existence of cells and organisms, the co-ordination of multicellular organisms and the species-specific process of embryonic development are ensured by a fine regulation of these conditions. It is these fundamental biological questions which the author tries to elucidate, and he does it in a spirit of criticism; he discusses the subject in an "open" manner by raising new questions up to the limits of our present knowledge. While mentioning several alternative possibilities, he expounds everywhere his own concept in the first place, and does it with a convincing logic based on carefully selected experimental material.

The book is written in a lucid style and provided with numerous instructive illustrations and diagrams. It is perhaps to be regretted that, while the material in respect of the cell is treated in all details, discussions concerning multicellular organisms (a subject coming nowadays in the foreground of interest) are of lesser extent. The work deals with the material from the angle of the biologist who is concerned with living cells and not from that of the biochemist. The conclusion at which the author arrives, representing the guiding principle of the work and illustrating his standpoint, is given in the following terms: "If . . . we still have a very imperfect understanding of differentiation, this, in my view, is because we have been looking too hard at the cell nucleus and not hard enough at the cytoplasm."

I. BERNÁD (Budapest)

ANNOUNCEMENT

The *First International Symposium on Cell Biology and Cytopharmacology* (sponsored by Dept. of Pharmacology, University of Milano, Italy and Dept. of Pharmacology, Laval University, Quebec, Canada) will be held in 1969 (July 7—11) at the "Fondazione Cini" Isola S. Giorgo, Venice, Italy.

Secretariat: Instituto di Farmacologia, Universitá di Milano—32, via Vanvitelli—20129 Milano (Italy)—Tel. 73.83.539.

Secretary of the Organizing Committee: Francesco Clementi, M. D.

Printed in Hungary

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A kézirat nyomdába érkezett: 1968. XII. 2. — Terjedelem: 11 (A/5) ív, 111 ábra (6 színes), 2 melléklet.

68.66722 Akadémiai Nyomda Budapest — Felelős vezető: Bernát György

A. KEVE

Das Vogelleben der mittleren Donau

Studia Biologica 7.

In German • 127 pages • Paper-bound

The author summarizes the accumulated ornithological knowledge in respect of the Middle Danubian Reach in connection with the international Danube research. Besides discussing nestling birds, migratory birds are also treated in detail. The study presents positive bromatological data which throw light upon provisionchain and its importance in agriculture.

T. HORTOBÁGYI

Phytoplankton Organisms from Three Reservoirs on the Jamuna River, India

Studia Biologica 8.

In English • 80 pages • Paper-bound

The micro-organisms of three reservoirs built on the Jamuna River are described. The knowledge of these is important both theoretically and from the point of view of water supply, irrigation as well as other practical uses of water. After describing the biotopes, the examination methods and the chemical conditions of the reservoirs, a detailed analysis of the micro-organisms is given. Thirty-seven new taxa are described. The illustrations are of special importance, all being original and of great theoretical value.

Evolutionary Trends in Fossil and Recent Hominids

Ed. by Gy. DEZSŐ and J. NEMESKÉRI

Symposia Biologica 9.

In preparation in English • Approx. 140 pages • Cloth

The Symposium arranged by the Biological Department of the Hungarian Academy of Sciences from 10 to 12 October, 1967, dealt with the trends of evolution of hominids. The papers of participants from eight countries survey the fields of palaenthology, antropology, human genetics and archaeology. This Symposium may attract special attention on account of the most recent pieces of information disclosed there in connection with the *Homo palaeohungaricus* of Vértesszőllős whose finds have been determined from various aspects of the evolutional theories.

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FERMENTATION OF PRIMYCIN

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(Received May 19, 1967)

Synopsis

Stable primycin-producing variants of the *Micromonospora galeriensis* have been isolated from a population heterogeneous as regards production of primycin, by culturing on a solid medium containing increasing primycin concentrations. Variant No. 4A/I—1 appeared to have the most favourable properties. In the course of the fermentation of this variant the effect of several parameters on primycin production was studied. By a qualitative and quantitative variation of the sources of carbon and nitrogen it was possible to find the most "productive" media for both shaken and submerged cultures. The selected strain utilizes fats and fatty acids as carbon sources or precursors in a manner favourable for primycin synthesis. The variant was found to be markedly aerobic. By ensuring the possibly best experimental conditions it was possible to obtain in shaken cultures and in metal fermentors of 1,000 l capacity a multiple of the amount of primycin produced by the original strain *Streptomyces primycini* n. sp. Some general biological problems connected with the production of primycin are discussed.

Introduction

In the course of their search for new antibiotics, VÁLYI-NAGY and co-workers [21, 22, 23] detected a species of *Streptomyces* which inhibited the growth of Gram-positive micro-organisms and mycobacteria even if highly diluted. The species was termed *Streptomyces primycini* n.sp. [14] and its active principle primycin [14]. The latter has the empirical formula $(C_{18}H_{37}O_8N)_6$. Its molecular weight was calculated 2,373, and was found by potentiometric titration to be 2,333 [15]. On the evidence of qualitative analyses primycin proved to be a guanidine arabinoside [16, 17] but its exact molecular structure is still unknown. In addition, recently FEHR and co-workers reported [3] that the molecule of primycin contains a primary amine, fifteen hydroxyl groups and two isolated double bonds. This and other results suggest that primycin may be a very large ring lactone. It is weakly soluble in water and polar solvents, and insoluble in non-polar ones. Its resistance to physical and chemical influences is high.

Pharmacological analyses and therapeutic experiments have proved the parenteral administration of purified primycin to be highly toxic to different laboratory animals [24]. Primycin has nevertheless aroused the interest of clinical specialists because (1) it is not — or is hardly — absorbed from the intestines and thus its oral doses in the g/kg range are tolerated; (2) it suppresses the resistant staphylococcal flora, (3) even its prolonged local application does not irritate the tissues or produce allergy [1, 7, 10, 28].

Table 1

Composition of media

Constituents in grams \ Culture medium No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Soy flour	20						20	30	50	30	50	20		20		20	40	
Amylum solani	20						25	35	50	35	50	25				30	40	
Amylum solubile													20					
Sucrose			30	30	10	30									30			
Glucose		10												10				
Peptone		5																0.5
Corn-steep liquor			10	30														
Yeast extract		20																
Killed yeast cells					10													
$CaCO_3$	5				1		5	5	5	5	5	5				5	5	
NaCl	3						3	3	3	3	3	3				3	3	
$NaNO_3$			2	2		2									2			
KCl			0.1	0.5											0.5			

K_2HPO_4				1		1												
KH_2PO_4	1	0.5	1		1		1	1	1	1	1	1	1	0.5	1	1	1	0.1
$Na_2HPO_4 \cdot 2\ H_2O$		0.5			1	0.5								1				1
$MgSO_4 \cdot 7\ H_2O$		0.5	0.5	0.5										0.5	0.5			
$FeSO_4$			0.1	0.1		0.1									0.1			
Digested filtered soy													40					
Palm oil																6	6	
Stearinic acid																	3	
Sunflower oil										10	10	20						
Meat extract (Difco)																		5
Agar-agar	20	15	15	15	20	15												20
Dist. water w/v			1	1		1												1
Tap water w/v	1	1			1		1	1	1	1	1	1	1	1	1	1	1	
Steriliz. autoclaved atm.	1.5	2	2	2	2	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Time of sterilization (min.)	20	30	20	20	45	20	20	20	20	20	20	20	20	20	20	60	60	20
pH after sterilization	7.2	7.2	7.3	7.2	7.8	7.2	7.3	7.3	7.3	7.3	7.3	7.3	7.0	7.0	7.0	7.2	7.2	8.2

After being isolated from its natural place of occurrence, the productivity of the primycin-producing strain was as low as 1—25 μg/ml. In order to make its production on an industrial scale possible, attempts were undertaken for the enhancement of the strain's productive capacity.

VÁLYI-NAGY and co-workers [25] were the first to point to the high variability of *Streptomyces primycini*. Relying on this finding, CSOBÁN and co-workers [2] tested the natural and artificial variants of the original strain for productivity, but failed to obtain a variant of considerably higher productive power that would have satisfied the requirements of industrial utilization.

As a result of the experiments to be described in the following, we succeeded in isolating from the wax moth *(Galeria melonella)* a micro-organism which produces primycin under the conditions described by us [23] in industrially utilizable quantities.

Material and method

Erlenmeyer flasks of 500 ml capacity, containing, in general, 100 ml nutrient medium, were used for the purposes of shaken cultures. In certain cases the amount of culture medium was reduced to 33 ml in order to ensure better aeration. The experiments were carried out at 28° C by means of a shaking machine which made 320 movements per minute with an amplitude of 37 mm.

The composition of the culture media employed in the experiments are shown in Table 1.

The strains used in the following experiments originated from the intestinal tract of wax moth larvae [21, 22]. They were preserved in sand cultures, and subsequently transferred to agar slants prepared from nutrient media Nos 1, 2 and 3 (Table 1). Incubation at 27° C lasted 120 to 140 hours. In order to check the purity and homogeneity of the strains and their variants, we transferred them to plates of nutrient media Nos 1, 3 and 4.

Media with the ordinary numbers as marked in the text (Table 1) were used for the plating out of the different variants.

SZYBALSKI's gradient-plate technique [19] was employed for the selection of primycin-resistant variants which are stable in regard to antibiotic production. The primycin preparation 144c—22 and media Nos 1, 2 and 6 were used for the gradient technique. We followed otherwise the instructions of SZYBALSKI and his associates with great accuracy.

In the experiments aimed at finding the nutrient medium that would prove best in shaken cultures for the stable antibiotic-producing variants of *Micromonospora galeriensis*, we varied the nitrogen (soy) : carbon (starch, lipids) ratio in the medium within limits that appeared to be favourable to primycin production (see media Nos 7, 8, 9, 10, 11 and 12 in Table 1). Media Nos 14 and 15 were used for the production of the inoculum needed for the experiments aimed at selecting the culture medium best suited for the growth of the primycin-producing variant 4A/I—1.

Redox dyes, e.g. 2,3,5-triphenyltetrazolium chloride [3] and methylene blue were employed for the determination of dehydrogenase activity in mycelia.

Culture medium No. 13 was used for the production of mycelial mass from which the "biomass" was obtained by centrifugation and washing with saline. The dehydrogenase activity was measured in this biomass, using Krebs—Ringer solution under anaerobic conditions. Dry weight was determined simultaneously. Samples selected for this purpose were evaporated in water bath and the residue was then dried at 105° C until stable weight.

The following formula served for the characterization and comparison of dehydrogenase activity in the various samples of mycelium:

$$a = \frac{1}{t \cdot m} 10^5$$

where

a = dehydrogenase activity;
t = time (in sec.) of the decoloration of methylene blue;
m = dry weight contents (in mg) of the biomass.

The agar-diffusion technique was used for the biological assay of primycin. *Bacillus subtilis* strain ATCC 6633 served as test organism. A chemically pure preparation of 144c—22, dissolved in a 1 : 1 : 2 mixture of butanol, methanol and water, served as the working standard of primycin. Solutions of 20 μg/ml were used. This standard solution may be kept at room temperature for several years without any loss of efficiency.

Results

In the first phase (1957—1963) of our search for variants of high primycin-producing activity we used the original *Streptomyces primycini* n.sp. These experiments were however unsuccessful. After having survived for a few generations, the productivity of the original strain and its variants sank below the practically utilizable level. We thought that turning back to the spores preserved at the time of the strain's isolation would result in active variants some of which might prove to be stable as regards production of primycin.

We found among the variants grown from the original sand cultures four colonies that showed a marked morphological difference from the original *Streptomyces primycini* n.sp. One of them proved to produce primycin, but the yield was not much higher than that obtained with the original strain.

The micro-organism, believed to be a new variant of *Streptomyces primycini* n.sp., was later subjected to careful examination, which showed that it was not identical with *Streptomyces primycini.* We termed it *Micromonospora galeriensis* n.sp. on account of its origin and morphological and biological properties.

We then seeded this strain on nutrient media Nos 1, 4 and 5, and, on morphological evidence, selected variants 1A, 1B, 3A, 3B, 4A, 4B, 5eA and 6eA. These were then cultured in shaken cultures on different nutrient media. Primycin was determined after 120 hours of fermentation. Results are shown in Table 2.

As seen in Table 2, media Nos 1 and 2 were the most favourable for the cultivation of the selected variants. Variant 4A — and to some extent also variant 1B — yielded a good crop of primycin.

Impelled by our earlier experiences [13] and relying on certain literary data [20, 27], we attempted to enhance the productivity of subvariants by promoting tolerance in respect of their own antibiotic. Accordingly, we transferred the productive variants to agar media containing a gradient of increasing primycin concentrations pursuant to SZYBALSKI's technique [19].

In the first gradient-plate series the concentration of primycin covered a range from zero to 1,000 μg/ml.

The variants were plated on gradient plates containing primycin from 0 to 1,000 μg/ml. Figures indicate the concentration level in the media from which the most primycin-resistant individuals were obtained.

As is evident from Table 3, the colonies were heterogeneous as regards sensitivity to primycin. The tolerance of the variants seemed to depend, among

Table 2

Effect of the composition of the culture medium on the amount of primycin produced by several variants of Micromonospora galeriensis

Concentration of primycin produced in shaken cultures 120 hrs after inoculation. Each figure represents the mean value of three measurements made in cultures grown in identically composed media

Culture medium No.	4	2	1
Variant	Primycin (μg/ml)		
1A	4	120	95
1B	10	420	100
3B	—	—	—
3C	15	330	350
4A	7	500	300
4B	3	100	50
5A	3	150	135
5aA	9	175	200
6A	9	350	260

Table 3

Primycin tolerance of the variants of Micromonospora galeriensis

Culture medium No.*	2	6	1
Variant	Maximum tolerance of colonies (μg/ml)		
1A	150	450	650
1B	200	450	450
3C	200	450	450
4A	250	450	500
4B	300	400	450
5aA	300	450	450
6aA	400	400	450

* Culture media representing the uppermost layer of the Szybalski plates.

others, on the composition of the culture medium. Organisms grown on soy-containing medium exhibited the highest degree of tolerance.

As the next step, we selected the most tolerant colonies and transferred them to plates containing primycin concentrations ranging from zero to 10,000 μg/ml.

As seen in Table 4, not even colonies grown in the presence of the highest primycin concentrations were equally sensitive to primycin, although they

Table 4

Selection of primycin-resistant variants of Micromonospora galeriensis in culture medium containing a 0—10,000 μg/ml gradient of antibiotic concentration

Variants	Approximate concentration-level of colonies showing maximum resistance (μg/ml)
1B/I	2,500
3C/I	3,000
4A/I	3,300
4A/II	2,800
4A/III	3,000
4A/IV	3,500
4B/I	2,500
5aA/I	3,800
6aA/II	2,800
6aA/III	4,300

Figures indicate the primycin tolerance of the most resistant colonies.

included an increased number of individuals which were able to grow at a concentration of 3,000 μg/ml or even higher.

Variants grown on the plates of the gradient 0—10,000 were then studied in shaken liquid cultures containing soy and starch. Although the variants isolated from the plates with the highest concentration of primycin did not prove to be remarkably productive, they were nevertheless the most stable in respect of productivity when tested in successive passages. Variants 4A/I–1, 4B/I–1 and 5aA–I surpassed the others inasmuch as their production of primycin remained at a practically constant level even after repeated passages at relatively long intervals.

At this stage of the investigations the question arose as to whether certain changes in the composition of the nutrient media or in other parameters of fermentation might induce the biologically standardized variants to increase their primycin production.

Preliminary experiments indicated a modification of the following parameters in the first place:

(1) composition of the culture medium (Table 5)

(2) (a) age and (b) amount of the inoculum (Table 6)

(3) time of fermentation (Table 7)

(4) O_2 supply of the cultures (Table 8)

It can be seen that each of the examined parameters influenced primycin production very considerably.

Table 5

Effect of the composition of the culture medium and of the amount of inoculum (vol./vol.) on the quantity of primycin produced by variant 4A/I—1

Designation of culture media	Amount of inoculum (vol./vol.)					
	20%		10%		1%	
	Designation of culture media used for the preparation of inoculum					
	14	15	14	15	14	15
7	220	33	200	230	350	320
8	250	170	280	260	250	400
9	220	125	250	300	260	430
10	320	100	390	130	500	490
11	330	90	220	120	650	860
12	250	80	380	150	500	800

The above figures indicate the μg/ml-value of primycin concentrations estimated in the different culture media. Each figure represents the primycin contents of the homogenized sample taken from three shaken cultures of the same group at the 120th hour of fermentation.

Table 6

Effect of the composition of the nutrient medium used for the preparation of inoculum and of the age of the inoculum on the production of primycin

Medium No.	Age of inoculum (hours)	Amount of inoculum (vol./vol.)					
		0.5%		1.0%		3.0%	
		Composition of nutrient medium used for the preparation of inoculum					
		15	14	15	14	15	14
11	24	195	280	3	350	2.5	340
	48	450	670	690	1400	800	470
	72	280	1	370	—	560	7
12	24	8.5	—	—	—	—	4.5
	48	0.1	600	320	340	330	1250
	72	420	—	770	—	400	—

Variant 4A/I—1 served as inoculum. The figures indicate the mean primycin concentrations (μg/ml) for three shaken cultures of the same group at the 120th hour of fermentation.

We wish to emphasize that the significance of the nominal age of the seed culture is far inferior to that of the biological activity or "biological age" of the cells contained in the culture. Biological activity of this kind is reliably indicated by the dehydrogenase activity of the cells [11].

Table 7

Effect of the time of fermentation on primycin production

Time of fermentation (hrs)	96	120	144	168	192
Variant	Concentration of primycin (μg/ml) in cultures				
5aA/I–1	320	500	480	640	530
4B/I–1	110	480	260	350	210
4A/I–1	280	330	900	610	420

The figures indicate mean values of primycin production estimated in three cultures of the same group. Culture medium No. 12 was used. 3%, 48-hr inoculum prepared in medium No. 15.

Table 8

Effect of aeration on primycin production

Degree of oxygen supply*	Primycin produced in medium No. 8 in the presence of 0.1%		
	margarine	palm oil	stearic acid
500/100	910	960	1260
500/33	1150	1060	2650

* *Numerator:* the volume (in ml) of the Erlenmeyer flask; *denominator:* the volume of the culture. The figures express the primycin concentration produced (μg/ml).

The inoculum was harvested from medium No. 14 after 48 hr incubation; its concentration was 1% (vol./vol.).

Table 9

Production of primycin in 1,000-litre metal fermentors

Time of sampling (hr)	Primycin produced (μg/ml)
35	35
47	238
59	191
71	1333
83	2280
95	2680
107	2900

For the purposes of inoculating the 1 m^3 fermentor variety 4A/I—1 was fermented in a tank of 100-litre useful capacity in nutrient medium No. 16. After 48 hrs of cultivation we inoculated medium No. 17 in the fermentor of 1,000-litre useful capacity with a 10% inoculum. Fermentation took place at a constant positive pressure of 0.4 atm. Aeration = 30 m^3/hr; temperature = 28° C; pH at 107 hrs = 6.9.

Fig. 1 shows a decrease in the dehydrogenase activity of the mycelium cells at 24 hrs, followed by a steep rise at 36 hrs. The peak was reached at 48 hrs, whereafter the activity dropped suddenly. It can further be seen that the time of maximum decoloration of the methylene blue, *i.e., 48 hrs,* was in good accord with the results of earlier experiments in which inocula of 48 hrs were found to give the best results. It is worthy of note that the sudden rise of primycin production coincided with a decrease in dehydrogenase activity.

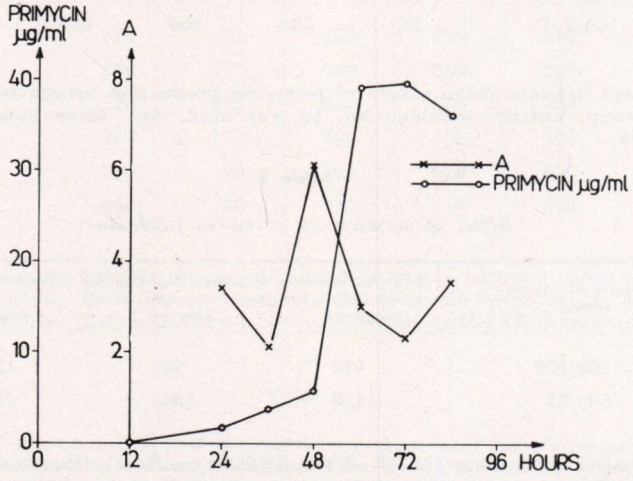

Fig. 1. The dehydrogenase activity in mycelium of the 4A/I–1 variant with ageing. The 4A/I—1 variant of *Micropolyspora galeriensis* in medium No. 13. Methylene blue final conc.: 0.05%; pH: 7.4

On the basis of the above-described experiments and observations we created conditions which enable us to produce primycin on an industrial scale. Table 9 shows the results of a typical experiment carried out in a 1,000-litre metal fermentor.

In this experiment, culture medium No. 16 was found to be the most suitable for mycelium growth, while medium No. 17 appeared especially favourable for a large-scale production of primycin. It yielded, under our experimental conditions, an average harvest of primycin amounting to 2,500—3,000 µg/ml.

Discussion

It has been noted above that the productive capacity of *Streptomyces primycini* n.sp., first isolated from the intestinal tract of *Galeria melonella,* was low. Neither the attempts of CSOBÁN and co-workers [2], nor our subsequent efforts succeeded in obtaining variants with constantly high productivity in

successive subcultures. On the other hand, repeated platings of the spores of the micro-organisms recovered from the wax moth resulted in the isolation of an organism, both morphologically and biologically different from *Streptomyces primycini;* we classified it, on the strength of its characteristic properties as belonging to the genus Micromonospora and termed it *Micromonospora galeriensis* n.sp.

By plating the strain and isolating its several morphologically different variants, it was established that the strain was heterogeneous in respect of primycin production. The growth of the majority of the colonies derived from the original spore population was inhibited by even low concentrations of primycin. Certain individuals of the spore population displayed, however, marked resistance to primycin; these resistant variants possessed a stable antibiotic-producing capacity so that it was justified to expect that, by an adequate modification of the fermentation parameters, they could be induced to produce extremely large amounts of primycin.

Observations made in connection with *Micromonospora galeriensis* provided renewed proof of the fact that the resistance of certain antibiotic-producing micro-organisms to their own products enables them to increase their production. WAKSMAN and co-workers [27] and, subsequently, HORVÁTH [5] had described this phenomenon, and MCDANIEL and HODGES [10] utilized it for the selection of *Streptomyces griseus* mutants of high streptomycin-producing capacity.

The fact that the composition of the culture medium has a considerable effect on the degree of primycin-resistance of *Micromonospora galeriensis* variants admits of several interpretations. We are possibly dealing here with a phenomenon like that described by SCHUMAN [13], who demonstrated that the composition of the culture medium remarkably influenced the development of "intraspecific antagonism". It is further possible that our seed spores were not uniform in respect of the degree of biological maturity. This depends on the degree of resistance developed by individual members of the population and also on the growth rate of the individual spores forming the colony. Presumably these factors determine the strain's sensitivity. All this depends on the composition of the nutrient medium.

As regards the factors promoting the productivity of 4A/I–1, the variant suitable for industrial utilization, our investigations concerning the quality of the inoculum prepared for the purposes of fermentation, further those regarding the composition of the culture media are noteworthy.

The dehydrogenase activity of differently aged mycelia of the variant 4A/I–1 made it evident that 48-hour cultures grown on a medium containing soy extract and starch included those biologically "youngest" hyphae which are most involved in the multiplication characterizing the first phase of growth. It seems to be significant that at this stage antibiotic production

was still dormant. Primycin production began later, when dehydrogenase activity was already decreasing.

It would follow from these phenomena that, at the early stage of its development, the primycin-producing organism displays increased sensitivity to its own antibiotic. A similar phenomenon was reported by INOUE [6, 7], viz. the streptomycin sensitivity of *Streptomyces griseus* considerably declined between hours 24 and 48.

Growth of the hyphal mass is proportional to the antibiotic production. On the other hand, ageing reduces the ability of hyphae to start, after being passaged, the proliferation of a hyphal population suitable for antibiotic production. This may be due to that in the aged inoculum the large amount of introduced antibiotic inhibits the growth of young hyphae. Inoculation with aged mycelia may moreover be unfavourable because, having gained access to the fresh medium, they may suddenly produce a large amount of antibiotic which prevents the multiplication of young hyphae in the entire culture at an early stage of fermentation.

NYIRI and LENGYEL [12] arrived at essentially similar results concerning the dynamics of oxytetracycline production. They found that the massive oxytetracycline production coincided with a decrease in the oxidoreductive activity of the *Streptomyces rimosus* strain.

The productive capacity of the examined strains including variant 4A/I–1 depends to a great extent also on the time of fermentation, on the quality and interproportion of the carbon and nitrogen sources in the medium as also on the O_2 supply of the cultures. It was found that the strain was well able to utilize fats and fatty acids for antibiotic production, a noteworthy phenomenon considering that the chemical structure of primycin presumably includes a saturated fatty-acid chain [18]. The constituents of the medium or their metabolites may be significant not only as carbon sources but also as precursor substances.

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PRIMYCINFERMENTATION

Auf festen Nährböden mit steigendem Antibiotikumkonzentrationsgradienten konnten die Verfasser aus der — im Hinblick auf die Primycinproduktion — heterogenen Population des Stammes *Micromonospora galeriensis* stabile Antibiotikum-produzierende Varianten isolieren, unter denen die Variante 4A/I—1 die vorteilhaftesten Eigenschaften aufwies. Bei der Fermentation dieser Variante wurde die Wirkung der Veränderung verschiedener Parameter auf die Primycinerzeugung untersucht. Durch qualitatives und quantitatives Variieren der Kohlenstoff- und Stickstoffquellen des Substrats wurde der bei Schüttel- und Tankfermentation optimale »produzierende« Nährboden ermittelt. Der untersuchte Stamm nutzt die Fette und Fettsäuren als Kohlenstoffquellen bzw. evtl. als Präkursoren für die Antibiotikumerzeugung gut aus. Die produzierende Variante zeigt einen hohen Bedarf an Luft. Bei optimaler Sicherung der erwähnten Faktoren sowie Einhaltung der angeführten Versuchsbedingungen konnte in Schüttelkulturen und in 1000 l-Metallfermentatoren das Mehrfache der Produktion des ursprünglichen Stammes (*Streptomyces primycini* n.sp.) erzielt werden. Abschließend werden die im Zusammenhang mit der Primycinfermentation auftauchenden Probleme besprochen.

ФЕРМЕНТАЦИЯ ПРИМИЦИНА

На плотной питательной среде, содержащей повышающийся градиент антибиотической концентрации, авторы изолировали стабильные, продуцирующие антибиотики варианты из популяции **Micromonospora galeriensis** гетерогенной с точки зрения производства примицина. Среди них наиболее благоприятными свойствами обладает вариант 4A/1—1. В процессе ферментации этого варианта изучали действие изменения различных параметров на продукцию примицина. Путем качественного и количественного варьирования источников углерода и азота в питательной среде был установлен субстрат, который при методе встряхивания или в танках оказался оптимальной «продуцирующей» средой. Изученный штамм хорошо использует жиры и жирные кислоты в качестве источников углерода или предшествующих соединений (прекурсоров) для производства антибиотического вещества. Вариант-продуцент показывает очень большую потребность в воздухе. При оптимальном обеспечении упомянутых факторов и при соблюдении указанных экспериментальных условий, при метода встряхивания и в 1000-литровых металлических ферментаторах можно было достигать производства антибиотического вещества, много раз превышающего продукцию антибиотика первоначальным штаммом (***Streptomyces primycini* n. sp.**). В заключении обсуждаются общие биологические проблемы, возникающие в связи с ферментацией примицина.

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STIMULATION OF THE PROCESSES OF SOMATIC EMBRYOGENESIS IN SOME PORIFERA AND COELENTERATA

III. EFFECT OF IRRADIATION ON THE FORMATIVE PROCESSES IN SPONGES AND COELENTERATA

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Synopsis

β-irradiation (preparation $^{90}Sr + {}^{90}Y$) was applied to the sponges *Leucosolenia complicata* MONT and *L. variabilis* HAECK, and to the hydroids *Clava multicornis* FORSCAL, *Laomedea flexuosa* HINKS and *Coryne loveni* M. SARS. *L. complicata* and *L. variabilis* are very resistant to β-irradiation. Doses of 25 kr, 50 kr and 100 kr are not lethal within 15—17 days. Doses of 450 kr do not kill *Laomedea flexuosa* and *Coryne loveni*. β-irradiation (2 kr—25 kr), inducing a disintegration of cell-systems, can provoke the processes of somatic embryogenesis and stimulate the initial processes of hydrant formation in *Laomedea flexuosa*. The authors connect the increase of radio-sensitivity with the increase of the organization complexity of the organism. The results of the irradiation of some *Planaria* and *Actinia* are discussed.

Introduction

In studying the processes of regeneration and somatic embryogenesis in sponges [3, 4, 5] and in Coelenterata [9], and the conditions which can accelerate or inhibit these processes, KOROTKOVA and TOKIN investigated the influence of β-irradiation on these animals characterized by a very low level of integration among the Metazoa.

Material and method

β-irradiation was applied to the colonial lime sponges *Leucosolenia complicata* MONT and *Leucosolenia variabilis* HAECK as well as to the hydroids *Clava multicornis* FORSCAL, *Laomedea flexuosa* HINKS and *Coryne loveni* M. SARS, inhabitants of the Barents Sea.

The source of irradiation was a preparation of $^{90}Sr + {}^{90}Y$ with an activity of 100 mC; the diameter of the slit was 20 mm. Aluminium foil 0.1 mm in thickness was used as filter. The animals to be irradiated were placed at a distance of 1—3 mm from the radioactive source (dose: 2,000 rad/min).

Control sponges and hydroids were placed in the same conditions of temperature, of the volume of water, etc., as the experimental animals.

Results

Sponges. Three series of experiments were carried out, the doses applied being 250 kr, 50 kr and 100 kr. For each series 50—100 oscular tubes of *L. complicata* and 20 tubes of *L. variabilis* were taken, all of them being removed from their prostrate parts. The observations were carefully registered.

Sponges of both species proved very resistant to β-irradiation. Doses of 25 kr, 50 kr and 100 kr fail to kill them within 15—17 days after the irradiation.

The rate of regeneration of the wall of the oscular tube was studied in 120 specimens of *L. complicata* after a small piece of the oscular tube was cut out. According to KOROTKOVA [4] a sponge completes the regeneration of a standard wound (about 1 mm^2) in its body wall on the 7th—10th postoperative day.

A piece of the body wall was cut out immediately after irradiation. Twenty-five kr exerted no marked effect on the regeneration rate, but doses of 50 kr and 100 kr protracted complete healing, by two days on the average. Presumably, the state of the choanocyte epithelium in the injured area is important for regeneration. According to KOROTKOVA [4], regeneration rate depends on the extent of the injury suffered by the choanocyte epithelium. The greater the number of choanocytes which, due to the injury, have lost their flagella and collars and passed into a dedifferentiated state in which they cannot take in any hard particles from the sea water, the slower is the final phase of the regenerative process, latter being connected with the choanocyte epithelium spreading all over the inner surface of the regeneration membrane.

In the irradiated tubes as well as in the control ones choanocyte epithelium was either changed along the whole oscular tube, or it was devoid of flagella in sections of various length. The number of sponges with more than a half of their choanocytes dedifferentiated was higher by 15% among the irradiated sponges than among the control ones. If the cut was made in the part of the body where the choanocytes were damaged, the regeneration was retarded.

As to the morphological peculiarities of the regeneration processes, no difference was found between the irradiated sponges and the control ones.

Coelenterata. Doses of 2 kr and 450 kr were used, the maximum time of irradiation being 4 hrs. The experiments were carried out on a great amount of material, ten or more colonies, with the same number of control animals in each series. *Clava multicornis*, on receiving a dose of 450 kr looked injured just after irradiation, their tentacles deformed, and bent, their gonophores obviously destroyed. In running sea water all of them died in 24 hrs after the irradiation, while the control animals remained normal.

A very simple and reliable method of determining whether the cells of a fresh-water hydra or of hydroid polyps are alive or dead can be recommended: trypan blue stains dead cells in a very short time, but is not absorbed by living ones.

Microscopic investigations showed that a dose of 25 kr produces no visible changes in a colony of *Clava*, and even a dose of 100 kr fails to kill it. But only special studies can prove whether or not these doses injure the organisms.

Similar results were obtained in the experiments on *Coryne loveni*. Doses less than 2 kr do not affect even the conidoblasts of *Coryne loveni*.

Laomedea flexuosa proved very resistant, the physiological conditions of its colonies remaining excellent for six days after an irradiation with 450 kr, no morphological changes being found in them as compared with the control animals. The physiological condition of the colony before the irradiation appears to be a very important factor for experiments show different results with one and the same dose. Some animals, on receiving this enormous dose (450 kr), seemed to be dead, their tentacles immobile. A day later some of the hydrants degenerated, the stolons, however, remained quite normal, showing no pathohistological divergence either in their ectoderm or endoderm. After another day the irradiated colonies were just like the control ones, except that the number of hydrants was reduced in some of the irradiated colonies. In the third series of experiments with the same dose no death occurred during the 8 days of observation. On the 5th day the number of pedicles having no hydrants, however, was greater in the irradiated colonies than in the control ones. Instead of forming new hydrants, the experimental colonies formed stolon-like "lashes", which will be described in details later.

Influence of β-irradiation on morphogenetic processes

Colonies of *Laomedea flexuosa* had all their hydrants cut off, their stolons and hydrorhizas were irradiated with 2 kr and, in another series of experiments, with 25 kr. Control animals, with their hydrants all cut off, were placed in the same conditions throughout the testing period. The next day the control colonies started forming new hydrants in stage I, their number attaining 62% of those having been cut off.* Colonies irradiated with a dose of 2 kr had 57% of such initial buds, whereas the colonies which had received 25 kr had even more buds than the control ones.

In another experiment, where the hydrants had been cut off before irradiation, the colonies irradiated with 25 kr 24 hrs before showed a more intense initial phase of somatic embryogenesis than in the control ones: 85% of hydrant buds in stage I in the experimental colonies as against 71% in the control ones. But on the second and the following days there appeared a certain inhibition, and differentiation was suppressed, probably due to the very disintegration of the cell systems which brings about the first stage of somatic embryogenesis.

In one of the series of experiments colonies of *Laomedea* were first irradiated with a dose of 2 kr and then all their hydrants were cut off. In three

* See Fig. 1 — The scheme of the stages, in the 2nd part of this paper [No. **20**(1) of this Journal].

days there were 33% of hydrants in stage I, 50% in stage II and 3% in stage III in the irradiated group, while the control group had 60%, 27% and 13% respectively. Five days after the hydrants had been cut off 59% of the developing hydrants of the irradiated colonies and only 35% of those of the control ones reached stage III. We suppose that the dose of 2 kr stimulates the initial processes of somatic embryogenesis which is connected with some disturbances in the normal integration of the organism, then these disturbances are overcome in a short period of time and the hydrants develop in a normal way.

The fact that β-irradiation in certain doses does not inhibit formative processes is confirmed by the experiments on the hydrorhizas of *Laomedea*. These were cut off from the colonies and then irradiated with 25 kr. The observations showed that the morphogenetic processes went on normally. In one case a new hydrant was formed in the place of the one cut off, and the growth of the hydrorhiza was seen at the ends.

The results of all the experiments with *Laomedea* leave no doubt that irradiation with 2 kr to 50 kr stimulates the initiation of somatic embryogenesis, provokes it, increases the number of hydrant buds, and makes the stolon-like formations grow. But in most cases, with doses of 25 kr and more, the initiated processes of somatic embryogenesis are interrupted, so that eventually the number of well-formed hydrants is greater in the control colonies than in the irradiated ones.

The fact that regenerative processes are retarded and at the same time somatic embryogenesis is provoked by β-irradiation is confirmed by experiments on *Clava*. Its hydrant pedicles were cut off and given additional traumas with a dissecting needle. A group of the pedicles were used as controls, other pedicles were irradiated with 25 kr, or 100 kr. Five days later the signs of somatic embryogenesis were found to be different in these three groups of pedicles. The control ones looked like smooth oval bodies (Fig. 1b, c), only one piece showing a kind of a growing tentacle bud (Fig. 1d). The pieces irradiated with 25 kr showed no signs of marked somatic embryogenesis. But the pieces which had received 100 kr presented quite a different picture, each piece having a new shape, with asymmetrically distributed buds of tentacles (Fig. 1e, f, g), like those seen by TOKIN in hydra.

The observations on regeneration proper are also of interest. Contrary to the current opinion, reparative processes in hydroids are at a very low level [9], the highest regenerative properties being found in *Clava*.

Some of the hydrants in *Clava* were cut off (Fig. 1a, A—B = the line of cut). One group of them operated in this way was taken as the control, the others were β-irradiated with a dose of 100 kr. In two days the surface of the wounds healed there being no difference whatever between the experimental and the control animals. Five days after the operation all the control animals formed new hydrants, much shorter than the ones which had been cut off.

Their apical end regenerated. After another day the newly formed hypostomas appeared quite normal, the new hydrants being active and capable of paralyzing small crustaceous which happened to come in contact with them. Histological investigations of hydrant fixed immediately after 6 days confirmed the complete regeneration of the hydrants in the control group.

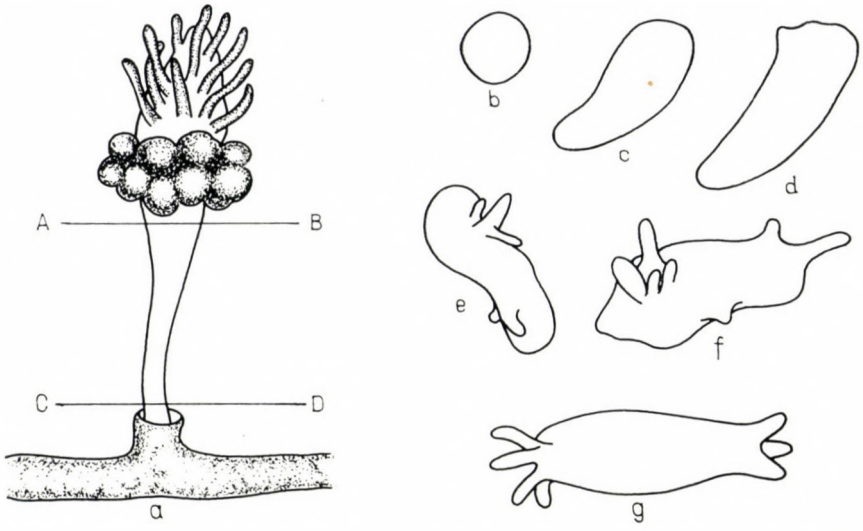

Fig. 1. Clava multicornis. Experiment with β-irradiation. *a* — scheme of the operation; *b, c, d* — control pedicles of *Clava* 5 hours after extirpation; *e,f,g* — initial stages of somatic embryogenesis on irradiated pedicles (100 kr)

The experimental animals were irradiated with a dose of 100 kr. On the fourth day after the irradiation small buds of future hydrants reduced in number and size as compared to the control group were seen. The next day the inhibition of regeneration in the irradiated group became even more clear, only one of many dozens of them having formed its hypostoma, while all the control ones had well formed hypostomas. Complete regeneration did take place in the irradiated animals, but a day later than in the control ones.

The next series of tests with *Clava* was carried out as follows. Animals were irradiated with 100 kr, on the next day the hydrants of both the irradiated and the control animals were dissected along all their length (Fig. 1a, C—D being the line of cut). Five days later (6 days after irradiation) the traumas healed in both groups. But it was not a complete or real restoration of the lost part of the body, the tentacles of many of the irradiated animals seemed dead, moving in a passive way, together with the moving water. Histological investigation confirmed that a complete regeneration had taken place neither in the irradiated nor in the control animals, though there was a certain difference

between the two groups. Cross-sections taken at various levels of the irradiated hydrants showed gaps in the normal layers of ectoderm and mesogloea, the gaps sometimes being about one third of the whole "circumference" of the hydrant and probably at the sites of the initial cut. In the gastric region, instead of a wall of normal structure a low bulging layer is to be found without cellular borders. Underlying the whole extent of this layer, a very thin membrane was formed, quite unlike the normal mesogloea, stretching over endodermal cells smaller in size than the cells in the uninjured parts of the hydrant. It is not clear whether these cells were brought there by the flow of endodermal fluids or they are the result from reproduction. In the former wound neither tentacles, nor gonophores are to be seen.

The cross-sections of the control hydrants show that their restoration is more complete, presenting a layer of ectodermal cells with distinct borders. These cells, however, are much smaller than those in the untraumatized parts of the ectoderm, the mesogloea being in the process of formation.

It may be concluded that β-irradiation inhibits regenerative processes.

In the experiments on *Laomedea* a disturbance of normal formative processes was observed, stolon-like, or, rather, hydrorhiza-like "lashes" being formed instead of hydrants. This was often the case with the colonies of *Laomedea* irradiated with 100 kr or more. Even these enormous doses failed to hinder the initiation of somatic embryogenesis, the development of the hydrants, however, was delayed and most of them was reduced and disappeared after a time. But one colony which had received a dose of 100 kr, developed three quite normal hydrants at the sites of the ones cut off. The structural regularity of the new hydrants was confirmed histologically. In this connection it should be mentioned here that according to PARK [6] X-irradiation with a dose of 25 kr or more is lethal for *Hydra littoralis*, but the buds which chanced to be irradiated sometimes survive and develop.

In 5 cases of the present experiments new hydrants were not formed. Instead, long "lashes", growing daily, appeared, and in one of the cases two lashes grew simultaneously in the place of the one cut off.* Microscopic study showed their structures to be similar, no matter whether irradiated with 2 kr or 100 kr. They have the structure of normal stolons, with the normal ectoderm, endoderm and periderm.

The formation of "lashes" after β-irradiation resembles the budding, well-known to embryologists and observed on many stalks of colonial hydroid polyps — blastostyles. These, however, have neither mouth nor tentacles and never attain the regular structure of a polyp. Every point of their body can produce a jellyfish by budding, so that a blastostyle usually has a body budding with gonophores.

* See Fig. 1a, b in the 2nd part of this paper [No. **20**(1) of this Journal].

Unfortunately, embryology cannot as yet solve the riddle of the formation of new polyps. According to TOKIN there are some physiological states of the organisms when normally a local or general disintegration of the cell systems can occur and cause budding or some other form of asexual reproduction.

Discussion

Modern theories on radiation injuries direct the main attention to proteins and nucleic acids as the largest "targets" among intracellular molecules. During irradiation they get the greatest amount of quanta. A biologist, however, cannot neglect the fact that the target hypothesis must be equally applied to plant cells and animal cells of any degree of complexity and of any level of integration, up to the organism of the man. The target hypothesis cannot explain the fact that there is a marked tendency to an increasing radiosensitivity with the increasing complexity of organization. To kill such poorly integrated organisms as plants (as compared with animals) enormous doses must be used (tens and hundreds of thousands and even millions of "r"). An amoeba survives 100 kr, while a rabbit dies in 30 days after being irradiated with a dose of 800 r. These facts point out that the main factor of a radiation injury is not limited by the effect on single cells and their nuclei. It is the effect on the regularities of the tissue life and organs and, probably, first of all of those whose normal functioning regulates and keeps in a normal condition the life of all other tissues and organs (the main regulating organs being the nervous system, blood-forming organs, etc.) which matters in an irradiation. Tissues and organs can be very radiosensitive, though the single cells comprising them can be rather resistant. The hypothesis of a "target" is powerless to explain the well-known facts of the regeneration ability of the mammals, amphibians and other animals being suppressed by ionizing radiations. Some changes occur in certain tissue and organ regularities, some changes in the architectonics of the tissues, which cannot be limited by the investigations on the molecular and cellular levels. TOKIN suggests that the constants of correlation existing between the proportions of cells in different stages of ontogeny are characteristic of certain tissues and organs. Since cells at different stages of their life cycle react differently to the same dose of an injuring factor, ionizing radiations may disturb these constants.

Ionizing radiations in due doses can induce such an "anarchization" and such a disintegration of tissues that the regenerative ability is lost. And it is the disintegration of tissues that can give rise to the morpho-physiological conditions for somatic embryogenesis. X-rays in doses exceeding 10 kr suppress regeneration in *Hydra oligactis* [10]. BABOCHKINA [1] confirmed these data in the laboratory of the Embryological Department, Leningrad, in 1949. Certain

doses proved to be able to suppress regeneration and at the same time provoke somatic embryogenesis, at least its initial stages. For instance, a dose of 10 kr stops, almost completely, the regeneration of the base and tentacles of a hydra, while the development of a whole hydra from a "circle" cut out of the gastric part outstrips the development of a whole hydra out of a similar circle which was cut out of a non-irradiated hydra. PARK also states that though doses of X-rays exceeding 25 kr are lethal for *Hydra littoralis*, pieces cut out of a hydra irradiated with a dose of 30 kr can develop into a whole hydra.

SEMENOVA-TYANSHANSKAYA [8] in the laboratory of Embryological Department (Leningrad University), on a suggestion of TOKIN, extensively studied the effect of X-ray irradiation on reparative processes in the planarian *Dugesia tigrina* GIRARD. She found that the whole of worms died in 10 days after being irradiated with 1,800 r, and that almost all worms perished by the 21st day following irradiation. But pieces taken out of the fore-part of their pharynx lived for 3 months (!) after receiving the same dose (1,800 r) of X-rays. An irregular process of somatic embryogenesis was observed, monstrous worms being formed with abnormal numbers of eyes. Whole planarians perish on being irradiated with a dose of 1,500 r but in the pieces cut out of the head parts of their body, after being X-rayed with this dose (1,500 r), processes of somatic embryogenesis were observed, the worms growing from them being quite viable.

POLTEVA [7] by a suggestion of TOKIN carried out extensive observations on the phenomena of somatic embryogenesis in actinian *Metridium senile fimbriatum* (VERRILL) and *Anthopleura orientalis* (AVERINCEV). Latter reproduces sexually, while former asexually, by liceration, i.e., from pieces of its base with small parts of the adjoining side wall of the body. When these "laceration fragments" were produced artificially, each fragment developed into a whole organism. This proved to be true for *Actinia equina* too, in spite of the fact that this species does not reproduce asexually. Analogous fragments of *A. orientalis* can survive for 3 months, without any morphogenetic changes. POLTEVA tried to induce somatic embryogenesis in *A. orientalis*, applying X-rays plus mechanical traumas as a disintegrating agents. Adult *A. orientalis* had their body wall notched in the middle part, and the wound area was irradiated for 10 min., with a total dose of 330 kr. Control actinians had similar wounds but no irradiation. Four irradiated actinians, out of 10, started to develop the oral disk with tentacles at the site of the irradiated incision (Fig. 2). These and other data of Polteva confirm the suggestion that local or total disintegration of tissues or of the whole organism is a precondition of somatic embryogenesis.

In this connection the experiments of ISAEVA [2] present a considerable interest, the experiments being carried out on planarian *Dugesia tigrina*, characterized by asexual reproduction. Under the action of an aqueous solution of the cancerogenic substance benzpyrene on a whole organism or on

pieces of it ISAEVA succeeded in provoking defective processes of somatic embryogenesis: several extra heads (up to 5) developed, or cylindrical or cone-shaped outgrowths, with eyes and brains in them, or several pharynges with

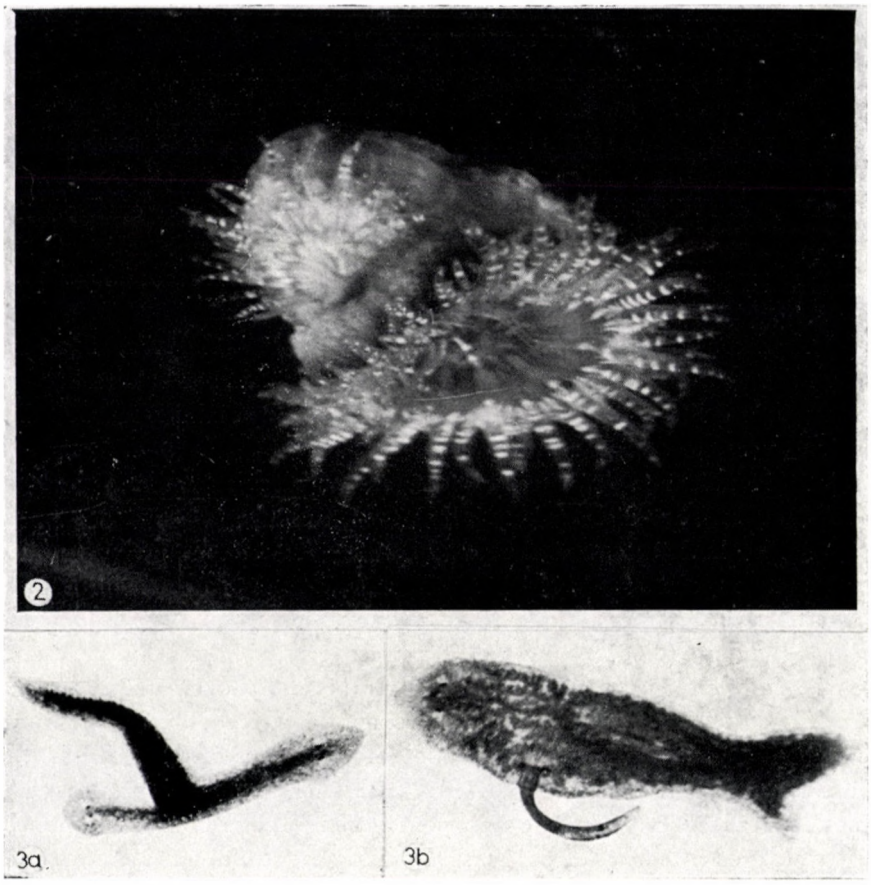

Fig. 2. Anthopleura orientalis. Development at the additional oral disk on the area of the irradiated incision (after POLTEVA, D. G., 1967)
Fig. 3. Somatic embryogenesis in *Dugesia tigrina. a* — caused by benzpyrene; *b* — caused by the "versene" (from ISAEVA, V. V., 1968)

various orientation, and other "monstruosities" (Fig. 3a). Versene, affecting intercellular relations and being capable of dissociating tissues into cells, also provokes somatic embryogenesis, which ISAEVA observed in her experiments (Fig. 3b).

Hence the authors expect the information and data given in this paper to be an interesting contribution to the theory of somatic embryogenesis and to radiobiology.

Conclusions*

(1) Authors performed the experiments on the basis of their theory of regeneration and somatic embryogenesis.

The genesis and the mechanism of the processes of regeneration and somatic embryogenesis are fundamentally different.

For regeneration proper to occur the organisms must preserve their relatively normal integration, while for somatic embryogenesis quite a different physiological basis is needed: a disintegration of the whole organism or of its parts, a disturbance of the "correlation locks" between cells and tissues, a radical morpho-physiological reconstruction of the original material.

The normal processes of asexual reproduction are preceded by a general or local disintegration as well.

(2) Authors gave the analysis of POLTEVA's, ISAEVA's, SEMENOVA-TYANSHANSKAYA's investigations on the provocation of somatic embryogenesis in *Actinia* and *Planaria* (mechanical traumas in the combination with X-ray irradiation, versene etc.).

(3) The influence of different cancerogenic substances (benzpyrene, ortho-tolidine and 3,3-dichlorobenzidine) on different species of Porifera was investigated by KOROTKOVA and GELIHOWSKAJA. The formation of numerous buds at the experimented oscular tubes in *Leucosolenia complicata* and *L. variabilis* was registered in a large proportion (up to 100%).

(4) Colchicine (0.001% and 0.01% concentrations) disturbing the normal regularities of cell systems, provokes somatic embryogenesis in *Laomedea flexuosa* HINCKS (Hydrozoa), its various forms depending on the peculiarities of asexual reproduction characteristic of this species of Coelenterata. One could observe the formation of hydrants, with their typical structure, or "hydrorhisolike fleshes", etc. Somatic embryogenesis can be provoked with mechanical disturbing of integrity of cenosark. The fragments (about 1 mm in diameter) being cut out the stolon *L. flexuosa* developed in the polyps. One fragment can produce two or more polyps.

(5) The β-irradiation (preparation $^{90}Sr + {}^{90}Y$) was applied to the sponges *Leucosolenia complicata* MONT and *L. variabilis* HAECK, and to the Hydroids *Clava multicornis* FORSCAL, *Laomedea flexuosa* HINCKS and *Coryne loveni* M. SARS. *L. Complicata* and *L. variabilis* are very resistant: doses of 25 kr, 50 kr and 100 kr fail to kill them in 15—17 days after the irradiation. Authors attempt to interpret this fact and the tendency to increase in the radiosensitivity with the increasing of the organization complexity of the organisms.

* See the papers in Numbers **19**(4) and **20**(1) of this Journal.

(6) Irradiation with 450 kr does not kill *Laomedea flexuosa* and *Coryne loveni*. β-irradiation (2—25 kr), inducing a disintegration of cell systems, can provoke the processes of somatic embryogenesis and stimulate initial processes of the formation of hydrants in *Laomedea flexuosa*.

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DIE STIMULATION DER SOMATISCHEN EMBRYOGENESE-PROZESSE BEI EINIGEN PORIFEREN UND COELENTERATEN

III. Über die Wirkung der Bestrahlung auf die Entstehungsprozesse an Schwämmen und Coelenteraten

Die Schwämme *Leucosolenia complicata* Mont und *L. variabilis* Haeck und die Hydroiden *Clava multicornis* Forscal, *Laomedea flexuosa* Hinks und *Coryne loveni* M. Sars wurden mit β-Strahlen ($^{90}Sr + {}^{90}Y$) bestrahlt. *L. complicata* und *L. variabilis* erwiesen sich gegenüber β-Strahlung als äußerst resistent. Nach einer Dosis von 25 kr, 50 kr und 100 kr gehen sie innerhalb von 15—17 Tagen nach der Bestrahlung nicht ein. Selbst Dosen von 450 kr hatten keine tödliche Wirkung auf *Laomedea flexuosa* und *Coryne loveni*. β-Strahlung von 2—25 kr, die eine Desintegration der Zellsysteme bewirken, können bei *Laomedea flexuosa* eine somatische Embryogenese provozieren und den Beginn der Hydrantenbildung stimulieren. Die Verfasser bringen die gesteigerte Strahlenempfindlichkeit mit der höheren Organisation des Organismus in Zusammenhang. Die Ergebnisse der Strahlenbehandlung einiger Planarien und Actinien werden besprochen.

СТИМУЛЯЦИЯ ПРОЦЕССОВ СОМАТИЧЕСКОГО ЭМБРИОГЕНЕЗА У НЕКОТОРЫХ PORIFERA И COELENTERATA

III. Материалы о действии ионизирующей радиации на формообразовательные процессы у губок и кишечнополостных

Изучено влияние β — излучения (источник $^{90}Sr + ^{90}Y$) на губки *Leucosolenia complicata* MONT. и L. *variabilis* HAECK и на гидроидных *Clava multicornis* FORSCAL, *Laomedea flexuosa* HINKS и *Coryne loveni* M. SARS. *L. complicata* и *L. variabilis* оказались весьма резистентными. Дозы 25 кр, 50 кр, 100 кр не приводили к гибели в течение 15—17 дней после радиации. Дозы до 450 кр не убивают *L. flexuosa* и *Coryne loveni*. β —излучение (2—25 кр), вызывая дезинтеграцию клеточных систем, может провоцировать процессы соматического эмбриогенеза и стимулировать возникновение процессов формирования гидрантов у *L. flexuosa*. Авторы связывают повышение радио-чувствительности с возрастанием сложности организации животных. Обсуждаются результаты воздействия радиации на некоторых планарий и актиний.

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HISTO-ENZYMOLOGICAL INVESTIGATIONS ON THE MUSCLE FIBRES OF A CEPHALOPODE (OCTOPUS VULG. LAM.)

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Synopsis

The cytochrome oxidase, succinic dehydrogenase, glucose-6-phosphate dehydrogenase and lactic dehydrogenase activities of the different muscles of *Octopus* were determined by biochemical and histochemical methods. The topographic distribution of the enzymes was found to correlate to the diameter of the muscle fibre. On the basis of this finding a comparison was made between the thin fibres of the *Octopus* and the red muscle of fish and mammals (rat) on the one hand, and between the thick fibres of the *Octopus* and the white muscle of fish and rat, on the other. A functional explanation of the results is suggested.

Introduction

Comparative study on muscle tissue of various molluscs has revealed a high enzymatic activity in the muscle of the *Octopus* as compared to the other animals investigated (Amphineures, Gasteropodes, Lamellibranchiates) [9]. This high enzyme activity prompted us to investigate this tissue from the histo-enzymologic viewpoint and compare it with muscles of fish and mammals.

Material and method

Histochemical examinations and enzymatic determinations were carried out on the muscles of the mantle, funnel and tentacles of *Octopus vulgaris* after removal of the epithelial layer.

Histochemistry. The removed muscles were rapidly frozen in liquid nitrogen and placed for 1—2 hours in dry ice, then sectioned in cryostat (SLEE) at —25° C (thickness of sections: about 20 μ). Duration of incubation was 10 min to one hour depending on activity.

The following enzymes were determined: *cytochrome oxidase* (CYO) [3] with and without cytochrome C; *succinic dehydrogenase* (SDH) with menadione and polyvinyl alcohol respectively, to increase activity and prevent its loss [1]; *glucose-6-phosphate dehydrogenase* (G-6-PDH) and *lactic dehydrogenase* (LDH) with and without *phenazine methosulphate* (PMS). The latter three enzymes were determined according to PEARSE [15].

Enzyme assays. The methods used were described by COOPERSTEIN and LAZAROW [4] (CYO), COOPERSTEIN and co-workers [5] (SDH) and BERGMEYER [2] (G-6-PDH and LDH).

The determinations of enzymes and proteins [10] were performed in supernatants obtained after homogenization in a Potter homogenizer and centrifugation at 10,000 g for 15 minutes at 3° C.

Results

The biochemical measurements have revealed that the *Octopus* has a higher cytochrome oxidase activity than other molluscs [12] but this activity is much weaker than that of mammalian muscles [4] (Table 1).

The mantle and the funnel were found to be richer in enzyme activity than the tentacle, the activity of the latter being similar to that of lower molluscs. The differences between the organs were less conspicuous regarding SDH, LDH and G-6-PDH activity. The latter activity was found to be always present in the molluscs in a concentration 10—20 times higher than that encountered in mammals, while LDH activity was found to be about 300 times less [9].

Table 1

Enzyme activities in muscles of Octopus

	N	CYO	SDH	G-6-PDH	LDH
Tentacle	4	0.039	0.031	9.0	18.4
Funnel	4	0.134	0.038	13.3	17.6
Mantle	3	0.132	0.059	13.9	32.6

Explanation

N = Number of animals
CYO = Δ log Ferrocytochrome C/mn/mg protein
SDH = Δ log Ferricytochrome C/mn/mg protein
G-6-PDH = μ Mole NADP reduced /mn/mg protein
LDH = μ Mole oxidized NADH /mn/mg protein

Morphology of the products of reaction

The CYO and SDH activities appear in the form of coloured grains localized mainly in the axis of fibres and sometimes in the sarcolemmal zone and in the interfibrillar spaces (Fig. 1). In longitudinal section the dense grains are arranged in simple regular rows or displaying a spiral-like form (Fig. 2). LDH and G-6-PDH activities appear in the form of homogeneous dark spots in the axis of fibres, where granulation is generally rare (e.g., in the funnel).

Topographic distribution of activities

Tentacle. For the topographic localization GUERIN's [7] terminology has been adopted (Fig. 3).

In transversal section the reactions of different muscular zones appear to be greatly differing.

The CYO and SDH activities were found to be relatively intense in the peripheral longitudinal layers of the four fascicles (Fig. 4). In these fibres the coloured grains appear simultaneously in the subsarcolemmal zone and in the interfibrillar spaces, having a spiral or oblique arrangement. Such coloured grains were never encountered in the central longitudinal zones where a slight activity was only noted. The transversal and oblique circular fibres and the

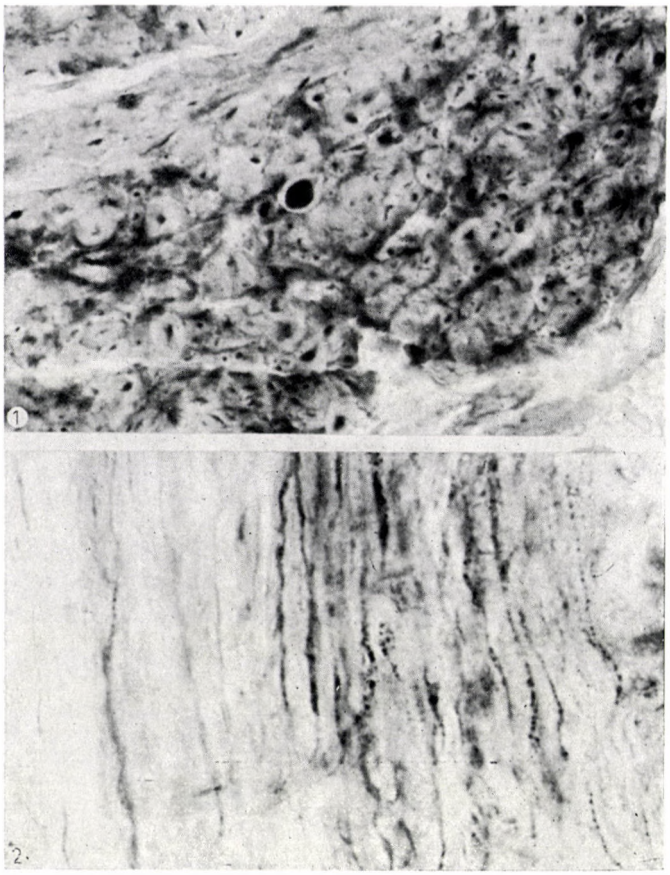

Fig. 1. Mantle. Cytochrome oxidase. Transversal section showing the intracellular localization ×730

Fig. 2. Funnel. Cytochrome oxidase. Circular zone. ×730

transversal trabeculae displayed an intermediary behaviour. If cytochrome C was omitted from the technique of Burstone, the reactions became even weaker and only persisted in the fibres of the peripheral longitudinal zones.

LDH (without PMS) and G-6-PDH activities had the same localization. On addition of PMS the reaction became stronger in all the fibres decreasing in this way the differences depending on the thickness of the fibres. The same was found in mammalian muscles [6, 18], and piscine muscles [11] (Table 2).

The distribution of the enzyme activities appeared to correspond to the diameter of the fibres. A marked difference in activity was noted between the thin fibres rich in enzymes, having an intensive "reactive axis", and the thicker fibres poor in enzymes, with a "reactive axis" constituted of 1 or 2 granules only.

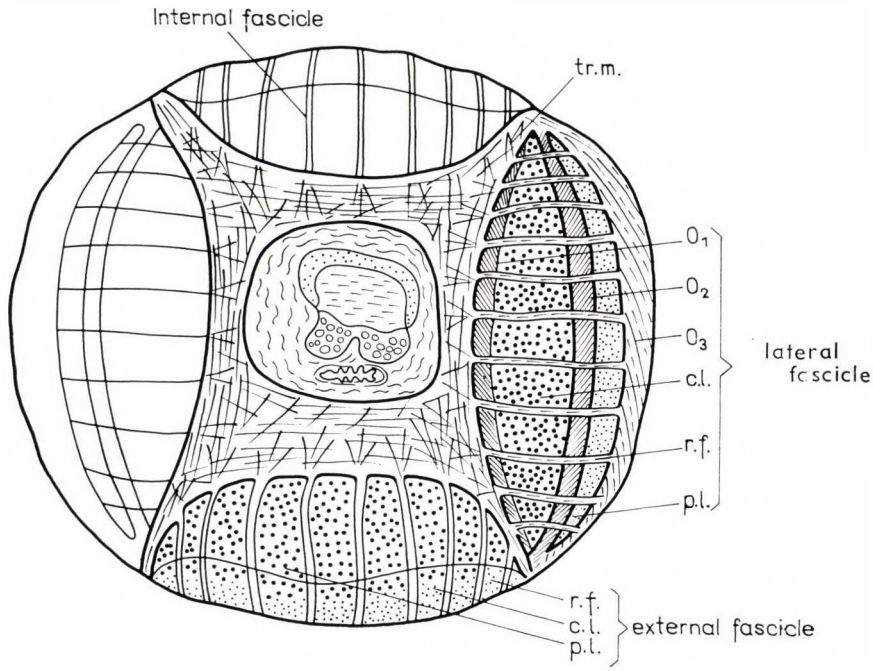

Fig. 3. Transversal section of the tentacle *(Octopus)*. c.l.: central longitudinal layer; p. l.: peripheral longitudinal layer; tr. m.: transversal musculature; O_1, O_2, O_3: oblique muscular layers; r.f.: radial fibres

Table 2

Comparison of enzyme activities of muscle fibres in a mammal (rat), fish (Myxine) and mollusc (Octopus)

		CYO	SDH	G-6-PDH	LDH	
					without PMS	with PMS
Rat	red fibres	++++[1]	++++[2]	o[2]	+++[3]	++++[3]
Fish *(Myxine)*	white fibres	+[1]	traces[4]	o[4]	traces[4]	++[4]
Octopus	thin fibres	+++	+++	+	++	+++
	thick fibres	+	+	o	traces	++

The differences in diameter and activity were found to be more conspicuous in the muscular zones of the lateral fascicles (small fibres in the longitudinal peripheral zone) than in those of external and internal fibres, where the diameters appeared to augment progressively from the peripheral to the central zones.

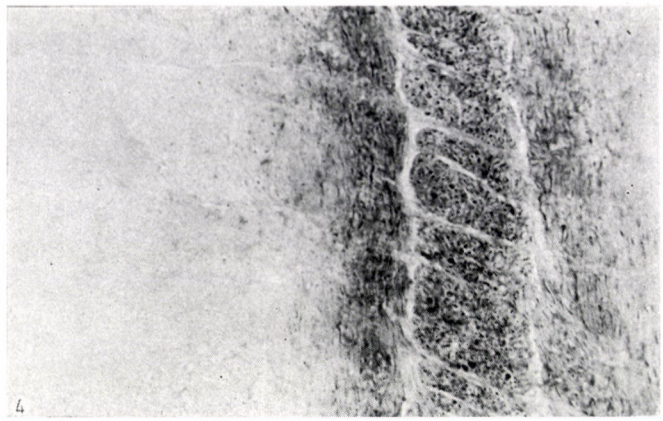

Fig. 4. Tentacle. Cytochrome oxidase. Strong reaction in the peripheral longitudinal zones. ×160

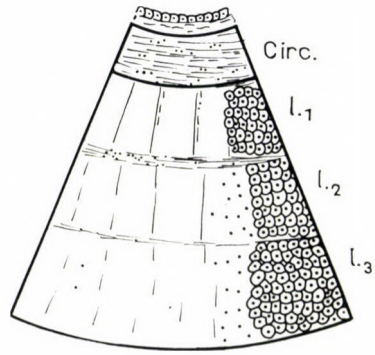

Fig. 5. Portion of the funnel (transversal section). Circ.: internal circular layer; l_1, l_2, l_3: longitudinal muscular layers

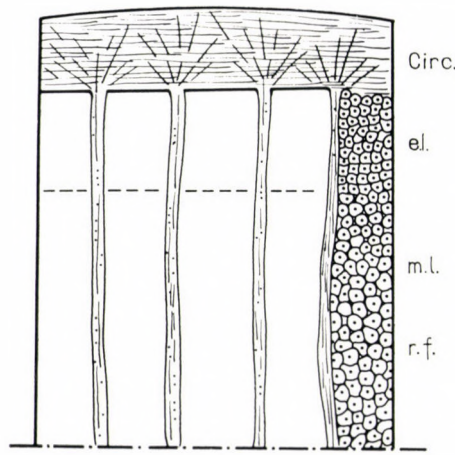

Fig. 6. Portion of the mantle '(transection). Circ.: circular muscular layer; r. f.: radial fibres; e.l.: external longitudinal layer; m.l.: median longitudinal layer

Funnel. The anatomical structure of the funnel having the same embryological origin as the tentacles, is similar to the muscle fibres of the latter organ (Fig. 5). The CYO activity demonstrable with cytochrome C, as well as SDH activity, were found to be stronger in the longitudinal zone 1, which is similar, regarding its fibres, to the peripheral longitudinal zones of the tentacles.

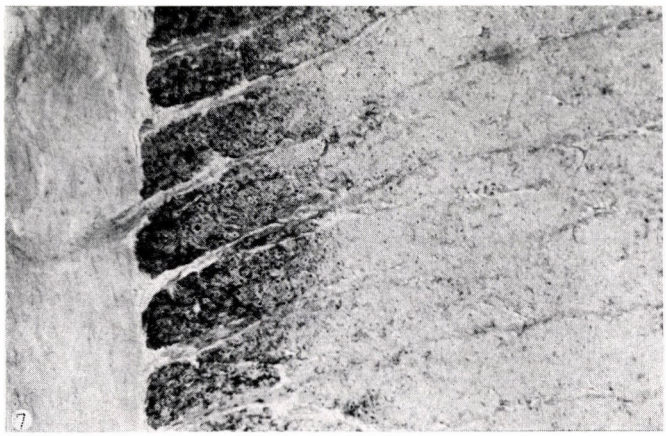

Fig. 7. Mantle. Cytochrome oxidase. Strong reaction in the zone constituted of thin fibres. ×120

The other muscular zones displayed but a slight activity, the internal circular zone showing here also an intermediary behaviour (Fig. 2). The general aspect regarding LDH (reaction reinforced by PMS) appeared to be identical with that of G-6-PDH, apart from a few granules in the "reactive axis" and under the sarcolemma of the most positive fibres of zones 1 and 2.

Mantle (Fig. 6). This important muscle is constituted of thin fibres rich in enzymes, localized on the two surfaces of the mantle, on both sides of a median zone composed exclusively of thick fibres poor in enzymes (Fig. 7). These longitudinal zones appeared to be traversed by radial fibres of very slight activity. The four enzyme activities studied in the mantle were found to have the same morphological characteristics as those of the tentacle.

Discussion

As early as in 1873 RANVIER recognized that the muscles of mammals and fish are composed of fibres having different physiological properties. There is, in fact, a mixture of thin red fibres and thicker white fibres. It is known that the red fibres having a tonic activity, have preponderantly an aerobic metabolism (though an anaerobic origin of ATP cannot be excluded), while the metab-

olism of white fibres capable of great and rapid efforts is predominantly anaerobic, requiring less energy. In addition to these fibres with distinct characteristics there are intermediary fibres as regards both their diameter and enzymatic activity.

Even a more conspicuous differentiation exists in the fish as has been noted by numerous authors. Some muscles are purely red, others are white, while a third group consists of mixed fibres in which all three types are found, grouped in separate zones.

In the majority of cases the slow fibres have a rhythmic activity and are localized at the periphery of the muscle bundles.

Octopus displays a similar arrangement of fibres having a mixed composition. This differentiation corresponds to the rhythmic respiratory movements of the mantle and funnel. The analogy with the diaphragm of mammals and certain muscles of fish is striking [13]. In the tentacle the peripheral localization of the thin fibres reminds that of the lateral muscles of fish permitting the maintenance of function even in the periods of rest. The deeper layers of large fibres are only involved in the reaction of flight or capture of prey. This interpretation seems to be confirmed by the finding that *Octopus vulgaris* possesses a complete cytochrome oxidase system.

Comparing the histo-enzymological features of different fibres of rat, fish *(Myxine)* and *Octopus* (Table 2), no essential difference was found apart from the presence of G-6-PDH in the small fibres of the polyp. It would be naturally premature to decide upon the intracellular localization of the enzyme, since we have very scanty information on the ultrastructure of these fibres. The reticular aspect of formazane or of the final product in the method of BURSTONE on CYO, does not indicate the hyaloplasmic or mitochondrial origin of these enzymes [17].

The central distribution of the coloured grains observed in all the fibres seems to be similar to that noted in the smooth muscle, though the red muscles of the *Myxine* [13] performing a rhythmic activity, display the same particularity.

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HISTOENZYMOLOGISCHE UNTERSUCHUNG DER MUSKELFASERN VON KOPFFÜSSLERN (*OCTOPUS VULG.* LAM.)

Die Cytochromoxydasen-, Succinodehydrogenasen-, Glukose-6-phosphatdehydrogenasen- und Lactatdehydrogenasen-Aktivität wurde in den verschiedenen Muskeln von *Octopus* mit biochemischen und histochemischen Verfahren bestimmt. Zwischen der topographischen Verteilung der Enzyme und dem Diameter der Muskelfasern fand sich eine Korrelation. Auf Grund dieser Befunde wurde ein Vergleich der dünnen Muskelfasern von *Octopus* und der roten Muskeln von Fischen und Säugern (Ratten) einerseits, und der dicken Muskelfasern von *Octopus* und der weißen Muskeln von Fischen und Ratten, anderseits, durchgeführt. Für die Deutung der Ergebnisse wird eine funktionelle Theorie in Vorschlag gebracht.

ГИСТО-ЭНЗИМОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ МЫШЕЧНЫХ ВОЛОКОН У ГОЛОВОНОГИХ (*OCTOPUS VULG.* LAM.)

Активность цитохромоксидазы, сукцинодегидрогеназы, глюкоз-6-фосфатдегидрогеназы и лактатдегидрогеназы была определена в различных мышцах *Octopus* с помощью биохимических и гистохимических методов исследования. Между топографическим рас-

пределением энзимов и диаметром мышечных волокон была найдена корреляция. На основе полученных данных проводилось сравнение тонких мышечных волокон *Octopus* и красных мышц рыб и млекопитающих (крыс) с одной стороны, толстых волокон *Octopus* и белых мышц рыб и крыс, с другой. Для объяснения полученных результатов выдвигается функциональная теория.

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ACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS OF ADENOHYPOPHYSEAL STH AND LTH IN ANDROGENIZED MALE AND FEMALE RATS

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Synopsis

Pituitary STH and LTH contents of female and male rats androgenized on the first or fifth day after birth were investigated by acrylamide gel electrophoresis. Extinction of the stain bound to the protein fractions proved to be a particularly good quantitative indicator of STH and LTH. Pituitary STH content increases, LTH content decreases in androgenized female rats. The degree of the changes induced depends on the timing of androgen administration. Results of the hormone assays are supported by the data of body weight and length of the animals as well as by the histological structure of the mammary glands. In males androgenization induces no such changes. Authors suppose that the changes induced are brought about by the effect of androgenization on the hypothalamus, but a role of a change in sexual steroid production cannot be excluded either.

Introduction

Testosterone administration to rats shortly after birth causes permanent endocrine changes at adult age. Female rats remain sterile, no ovulation can be observed in the ovaries and corpora lutea are not formed [2, 4, 11, 12, 13, 25, 36, 38]. Although testosterone administration does not interfere with fertility in male rats, the adult weight of the testicles and accessory sexual organs of the treated animals is smaller than in normal animals [15, 16, 40, 41].

Data on pituitary gonadotrophin *production* (secretion and release) of androgenized rats are contradictory. Some authors found an increased hypophyseal LH content [38], others reported a decreased hormone production [3, 10, 31], or an unchanged one [42, 43]. FSH production is generally believed to be diminished [14, 15, 21], but reports on an increased production have also been published [31].

There are considerably fewer data on the production of the other pituitary trophic hormones in androgenized rats. Testosterone administration in the early postnatal period does not really affect ACTH production [27, 37, 39]. Studies on TSH indicate a decreased production [5, 19]. No deciduoma can be provoked in androgenized animals [44], and LTH production was found to be decreased [22].

Our earlier studies with starch gel electrophoresis [1, 32] indicated that androgenized female rats show an increased pituitary STH content [23, 24]. These findings are supported by data published on the body weight of experimental animals in the studies mentioned above. SWANSON and VAN DER WERFF TEN BOSCH [40] made a detailed analysis on the growth and body weight of androgenized animals and found an increase in body weight.

The present investigations were aimed at analysing in detail the pituitary STH and LTH content in androgenized rats with regard to the timing of androgenization.

Material and method

White rats of the "R-Amsterdam, Hungary" strain were used throughout. The rats were kept on a standard diet. Several rats in each litter received 500 μg testosterone phenylpropionate (Retandrol, Richter, Budapest) dissolved in 0.1 ml oil immediately after birth, others were injected with the same dose 4 days after birth (i.e. on the fifth day), the remainder served as control.

Controls and androgenized rats were killed by decapitation at the age of 5 month. Six days before the killing after a 16-hour fasting, body weight and length of the animals were taken in ether narcosis. Their endocrine organs and mammary glands were weighed, fixed in 4% formaline and examined in histological sections stained with haematoxylin–eosin. The adenohypophysis was separated from the neurohypophysis, weighed with a precision of 0.2 mg and homogenized at 0 to +5° C in 0.05 *M* phosphate buffer of pH 7.4. The homogenate was diluted with large pore gel solution to contain 15 mg wet hypophysis tissue in 1.0 ml. After centrifugation 0.2 ml of the supernatant was transferred to the acrylamide gel column prepared as described by ORNSTEIN [33] and DAVIS [7]. Electrophoresis was carried out over 120 minutes, starting at 3.8 mA/tube. The electrophoretograms were stained with Naphthalene Black 10 B (C. I. 20470; Reanal, Budapest) diluted in 7% acetic acid and 1 g stain was used for 100 ml. Staining was followed by differentiation for 96—120 hours, whereafter the STH, albumin and prolactin fractions were cut off and ground in a mixture containing 0.5 ml *N* NaOH and methanol in a ratio of 1 : 1. After elution for about 30 minutes the suspension was centrifuged and the extinction of the supernatants determined with a "Beckman Spinco 151" type spectrocolorimeter at 610 mμ wavelength.

In some cases the unstained gel columns were cut along the bands, ground in 0.6 ml distilled water, kept for 24 hours at 4° C and centrifuged at the same temperature. Prolactin activity of the supernatant was determined by the pigeon crop micro-method [20]. Our results on prolactin activity of the single fractions will be published elsewhere.

The results of the experiments were evaluated by Student's "t" test.

Results

Tables 1 and 2 as well as Fig. 1 show the results.

Table 1 shows a significant increase in body weight and length (nose-anus, nose-end of tail) of the females androgenized on the first day. The values for females androgenized on the fifth day stand between the former group and the controls, whereas there is no difference among the groups of male rats.

Table 2 shows the extinction of the stain obtained from the protein fractions with STH or LTH activity. The degree of extinction is proportionate to the somatotrophin and prolactin content of the adenohypophysis, respectively. The hypophysis of the male controls had the highest STH content and this

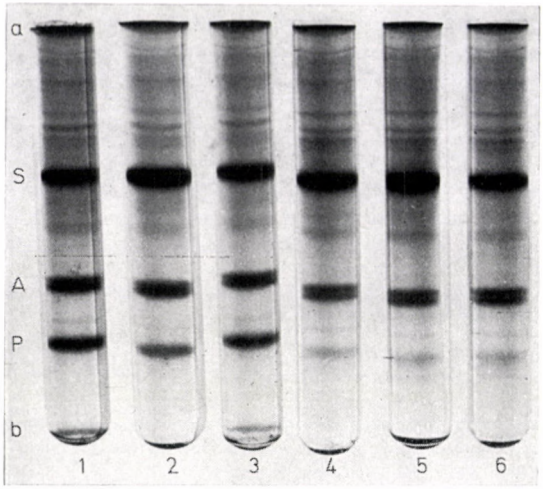

Fig. 1. Electrophoretograms of control and androgenized rat adenohypophysis. S = fraction of STH activity; A = albumin; P = fraction of prolactin; a = start; b = front. 1 = control female; 2 = first-day androgenized female; 3 = fifth-day androgenized female; 4 = control male; 5 = first-day androgenized male; 6 = fifth-day androgenized male. Figure 1 shows that in the anterior pituitary of the female androgenized on the first day STH activity band is more intensive, LTH activity band is less intensive than in the control female. There is no significant difference among the groups of male rats

Table 1

Comparison of body weight and length of control and androgenized rats

Groups	Sex	Number of animals	Body weight (g)	Length of animals (cm)	
				nose-anus	nose-end of tail
1. Control	♂	8	237±14*	21.8±0.35	38.8±0.52
2. First day androgenized	♂	15	242±21.0	21.7±0.17	39.1±0.25
3. Fifth day androgenized	♂	14	256±10.0	21.9±0.11	39.4±0.45
4. Control	♀	10	171± 6.4	19.5±0.27	35.5±0.32
5. First day androgenized	♀	22	202± 6.0	20.5±0.15	37.2±0.76
6. Fifth day androgenized	♀	12	188± 3.8	20.2±0.13	36.8±0.18

*Standard error of the mean	4.—5.**	$P < 0.01$	$P < 0.01$	$P < 0.01$
	4.—6.	$0.05 > P > 0.02$	$0.05 P > 0.02$	$P < 0.01$
** Probability	5.—6.	$0.1 > P > 0.05$	$P > 0.5$	$P > 0.5$

was hardly influenced by androgenization. Among the females those androgenized on the first day were the richest in hypophyseal STH, yet poorer than the male controls. The control females and the females androgenized on the fifth day had still less pituitary STH.

Table 2

Quantitative changes in certain protein fractions of pituitary homogenates separated by acrylamide gel electrophoresis

Groups	Sex	Age of animals	Weight of hypophysis	Number of analysis	Extinction[0] × 10^3		
					Fraction of STH activity	Albumin	Fraction of LTH activity
1. Control	♂	140 days	8.8±0.4	6	1114±162*	177± 7	58± 2
2. First day androgenized	♂	140 days	8.1±0.27	6	988±123	163±14	51± 4
3. Fifth day androgenized	♂	140 days	8.2±0.46	5	825±110	181±14	54± 4
4. Control	♀	140 days	10.4±0.26	12	474±37	227±17	174±11
5. First day androgenized	♀	140 days	10.8±0.4	11	784±42	256±21	93±10
6. Fifth day androgenized	♀	140 days	9.2±0.33	8	619±38	249±12	122± 5

* Standard error of the mean.
** Probability
[0] 3 mg wet weight of hypophysis

1.—2.**	$P > 0.5$	$P > 0.5$	$P > 0.5$
1.—3.	$P > 0.1$	$P > 0.5$	$P > 0.1$
1.—4.	$P < 0.01$	$P > 0.5$	$P < 0.01$
1.—5.	$0.02 > P > 0.01$	$P > 0.5$	$0.05 > P > 0.02$
2.—5.	$0.1 > P > 0.05$	$P < 0.01$	$P < 0.01$
4.—5.	$P < 0.01$	$P > 0.5$	$P < 0.01$
4.—6.	$0.02 > P > 0.01$	$P > 0.2$	$P < 0.01$
5.—6.	$P < 0.01$	$P > 0.5$	$0.05 > P > 0.02$

Prolactin content shows the well-known sex differences. Androgenization has no action on the pituitary prolactin content in males, and induced a significant decrease in females. The histological structure of the mammary glands of female rats was not influenced by androgenization.

Discussion

Gel electrophoresis is a method widely adopted to study protein fractions of the adenohypophysis. The spot location of the fractions of STH and of LTH activity has been established by several authors [6, 9, 17, 26, 28, 29, 30]. The fraction indicated as LTH was controlled in the present experiments, too. We could prove that the degree the extinction is directly proportionate to the amount of the hypophysis electrophoretized or to the amount of purified prolactin (Panlitar, Armour). These findings are in accordance with electrophoretic analyses made on the hypophysis of rats in different physiological and pathophysiological conditions [23, 24].

We attribute the increased hypophyseal STH content in androgenized female rats to an increased production and not to an accumulation of the hormone secreted, i.e., to a decreased release. Figures of body weight and length of androgenized females confirm this concept. High STH production is characteristic of males. An increased hypophyseal STH content in androgenized females, together with an increased body weight and length indicate a shift to the masculine of the secondary sexual characteristics. The degree of the changes depends on the timing of testosterone administration, being more pronounced for the animals treated on the first day than for those androgenized on the fifth day.

"Masculinization" of testosterone treated females is indicated by a decrease of the hypophyseal LTH content, too. Our present findings correspond to the results obtained earlier by the pigeon crop micromethod [22] and are in agreement with the observations of KUMARESAN and TURNER [18] on mammary glands. These authors reported that a single injection of testosterone administered in the neonatal period induced an 18% decrease in the growth of mammary ducts and a 33% decrease in the lobulo-alveolar development by the 120th day of age.

Disturbances in the sexual functions in androgenized females have been ascribed to the lesions induced in the hypothalamus. The continuous and decreased LH production can be fully explained by the desensibilization of the sexual-steroid-sensitive neurons in the anterior pituitary [8, 21, 34, 35]. But a decreased gonadotrophin production is most probably related to lesions in other hypothalamic areas, too [21], as supported by the decreased gonadotrophin production in male rats androgenized on the first day.

Results of our present experiments also indicate that early androgenization affects the hypothalamus. We suppose that the increased STH and decreased LTH production is due to a shift to masculine in the regulating mechanism of the hypothalamus. It is most probable that a suitably timed androgen treatment affects all pituitary centres whose function will develop in the male and female organism in different ways, and a subsequent masculinization ensues in females.

The results of our experiments do not exclude the possibility of an indirect effect of early androgenization either. As disturbance in gonadotrophic hormone secretion affects production of sexual steroids, it is quite possible that the change in steroid production has also a role in changing pituitary STH and LTH production.

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ANALYSE DES STH- UND LTH-GEHALTES DER ADENOHYPOPHYSE VON ANDROGEN-BEHANDELTEN WEIBLICHEN UND MÄNNLICHEN RATTEN MITTELS AKRYLAMIDGEL-ELEKTROPHORESE

Der STH- und LTH-Gehalt der Adenohypophyse weiblicher und männlicher Ratten die 1 Tag bzw. 5 Tage Androgen erhielten, wurde mittels Akrylamidgel-Elektrophorese bestimmt. Die Extinktion des durch die — mittels Elektrophorese getrennten — Eiweißfraktionen gebundenen Farbstoffs erwies sich als guter Indikator der Hormonmengen. In der Hypophyse androgenisierter weiblicher Ratten erhöht sich der STH-Gehalt, während die STH-Konzentration abnimmt. Das Ausmaß der Änderung hängt vom Zeitpunkt der Androgenadministration ab. Die Ergebnisse der Hormonbestimmungen wurden auch durch Körpergewicht- und Körpermaß-Messungen, ferner durch die histologische Untersuchung der Milchdrüsen bekräftigt. An männlichen Ratten bewirkt die Androgenbehandlung keine diesbezüglichen

Änderungen. Die abweichenden Hormonkonzentrationen werden mit der ausgeprägten Wirkung der Androgene auf den Hypothalamus in Zusammenhang gebracht, doch auch die Möglichkeit wird erwogen, daß daran auch die veränderte Sexualsteroidsynthese beteiligt ist.

АНАЛИЗ СОДЕРЖАНИЯ СТГ И ЛТГ В АДЕНОГИПОФИЗЕ КРЫС САМЦОВ И САМОК, ПОЛУЧИВШИХ АНДРОГЕНЫ, МЕТОДОМ ЭЛЕКТРОФОРЕЗА НА ГЕЛЕ АКРИЛАМИДА

Авторы проводили анализ содержания СТГ и ЛТГ в аденогипофизе крыс-самок и самцов, получивших андрогены на 1-ыи или 5-ыи день после рождения, методом электрофореза на геле акриламида. Было установлено, что поглощение света красителем, связанным с фракциями белков, отделенными методом электрофореза, представляет собой хороший индикатор количества исследовавшихся гормонов. В гипофизе крыс самок, получивших андрогены, содержание СТГ повышается, в то время как содержание ЛТГ понижается. Величина изменения зависит от срока введения андрогена. Результаты определения содержания гормонов подкрепляются весом и размерами тела, а также гистологическим исследованием грудных желез. У самцов дача андрогенов не вызывает изменений такого направления. Авторы объясняют указанные изменения выраженным действием андрогенов на гипоталамус, однако, не исключено, что в этом участвуют также изменения синтеза половых гормонов.

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EFFERENT PATHWAYS OF THE OPTIC TECTUM IN THE FROG

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Synopsis

With the aid of axon degeneration technique three efferent pathways of the optic tectum can be distinguished in the frog. The rostral-oriented pathway traverses forward in the base of the mesencephalon and diencephalon, crosses to the other side in the postoptic commissure and returns to the mesencephalic gray matter on the contralateral side. The pathway establishes connections with the nucleus isthmi on the same side, with the tegmentum mesencephali, the pretectal region, and the thalamus on both sides, and with the retina on the contralateral side. The caudal-oriented pathway corresponds to the tractus tectobulbaris and -spinalis. The greater part of the pathway decussates in the commissura ansulata, and both the uncrossed and crossed limbs can be followed to the lumbal segments of the spinal cord. Along its course in the medulla oblongata, the pathway gives origin to several aberrant fibres, which enter the gray matter surrounding the motor nuclei of cranial nerves. At the bulbo-spinal border the descending fibres rapidly decrease in number, and the remaining fibres terminate in the ventral horn and in the zona intermedia of the spinal cord. The third efferent pathway interconnects the two tecta (commissura tecti diencephali and mesencephali). These commissural fibres terminate in the 2nd to 6th layers of the contralateral tectum opticum.

Introduction

Very little is known about the efferent projections of the optic tectum in frogs. Data in the literature are mainly based on the investigations of GAUPP [5], ARIËNS KAPPERS and co-workers [1], HERRICK [8] and RÖTHIG [19]. With the aid of various silver impregnation techniques the authors were able to show direct efferent projections from the tectum on the thalamus, hypothalamus, habenular nuclei, isthmic nucleus, cerebellum, nuclei of the extraocular muscles and on the medulla oblongata. In addition to these projections a number of commissural tracts have been described between the two tecta.

The histological techniques used for the investigations are apt only to show solid tracts and bundles, but do not provide information about central pathways composed of loosely scattered fibres intermingling with a number of other fibres of various origin. In view of this techniqual deficiency we have no reliable data of, e.g., spinal projections from the tectum or of the much discussed tecto-retinal efferent connections. Since the visual system of the frog has recently become the centre of a number of different physiological and behavioural investigations, the necessity arises to obtain more detailed knowledge about

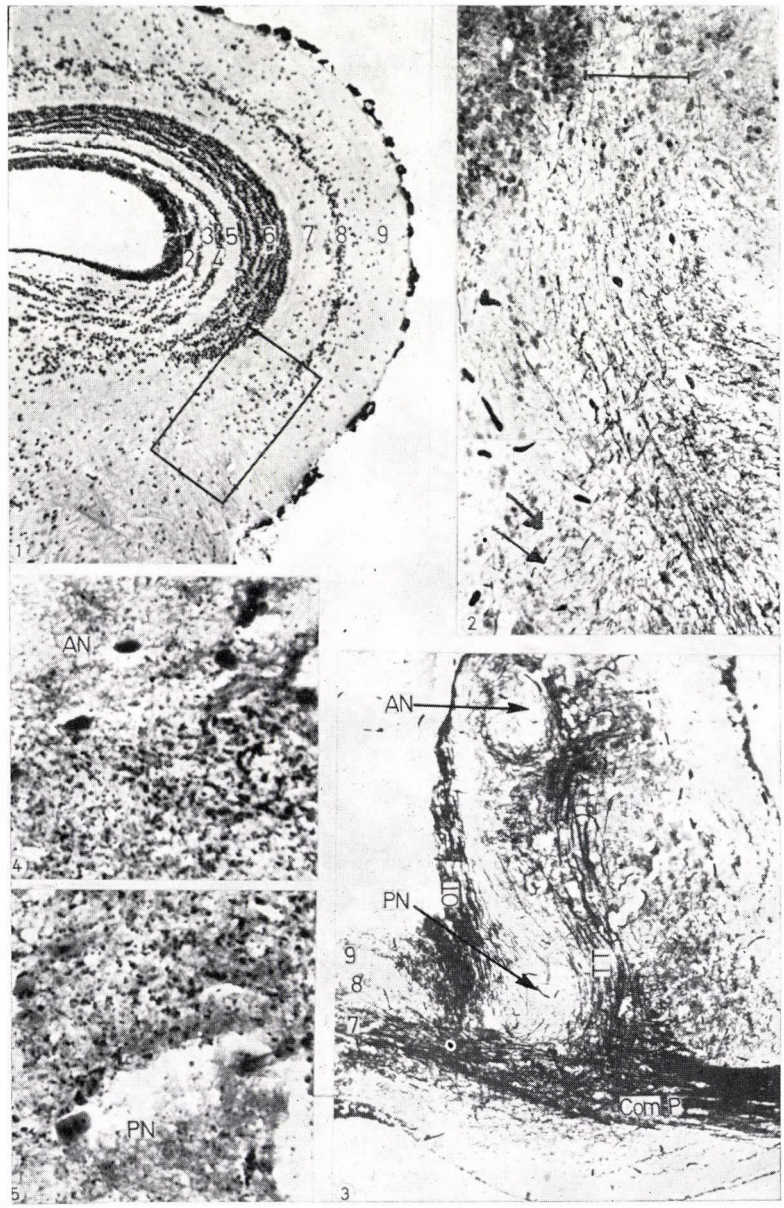

Fig. 1. Transversal section of the lateral half of a normal optic tectum stained with Klüver–Barrera technique. The numbers indicate the stratification of the tectum. The enframed part corresponds to the area shown in Fig. 2. ×48

Fig. 2. The lateral border of the optic tectum (as indicated in Fig. 1) on the side of operation. Large number of degenerated fibres can be seen to leave the tectum through layer 7 (indicated by a horizontal bar). The majority of fibres turn laterally (right in the picture), some of them indicated by arrows turn medially and enter the mesencephalic gray matter. Nauta-Gygax silver technique. ×108

Fig. 3. Horizontal section of the diencephalon of a normal frog. Gallyas silver technique to

the anatomy of this system. The present paper is a part of this attempt and gives an account of efferent connections of the optic tectum investigated with the axon degeneration method.

Material and method

The investigations were done on 33 adult frogs *(Rana esculenta)*. The optic tectum on the one side was either completely or in greater part removed. The operated animals were kept alive for 3 to 23 days. A survival of 14 days proved to be most advantageous to show degenerated axons. The animals were perfused rapidly with physiological saline under anaesthesia and subsequently with 10% neutralized formol solution. The brains and spinal cords were removed and kept in formol of similar concentration for a period longer than two weeks. Before cutting with the freezing microtome the materials were embedded in 25% gelatin solution and the blocks were treated with 10% formol in the refrigerator for 24 hours.

To show degenerated axons in the frog the Nauta-Gygax technique [18] was slightly modified. The concentration of the phosphomolybdenic acid and that of the potassium permanganate was increased to 1% and to 0.5%, respectively. The ammoniacal-silver solution was also slightly altered: 1 g $AgNO_3$, 20 ml distilled water, 10 ml 96% ethanol, 1.5 ml strong ammonia water and 1.6 ml 2.5% sodium hydroxide. In addition to this method, two other types of Nauta modifications described by GRANT and WESTMAN [6] and by FINK and HEIMER [3] were employed. The latter proved to be very useful to show degenerated terminals after a 5-day survival period. The brains of a few intact animals were treated to show myeline sheets with Gallyas' [4] silver technique.

Results

For orientation in the optic tectum the scheme of stratification published earlier [14] is used. According to this, the optic tectum consists of 9 alternating cellular and plexiform layers. Starting from the ependyma, layers 2, 4 and 6 are thickly packed cellular layers being separated from one another by plexiform layers 3 and 5 (Fig. 1). This part of the tectum corresponds to the *stratum album et griseum periventriculare* in early descriptions [1, 5] and its cellular layers can be directly followed to the central gray matter surrounding the cerebral aqueduct. Neurons in this part show a pear-shaped and pyramidal form with long shaft dendrites extending towards the surface. Layer 7 *(stratum album centrale)* is void of cells containing predominantly thick myelinated fibres. This layer directly leads into the basal white matter of the mesencephalon, which contains ascending and descending pathways of various origin and the dendritic tangle of mesencephalic neurons. Layer 8 *(stratum griseum cen-*

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show myelinated fibres. *AN* = anterior-superior nucleus of the lateral geniculate body; *PN* = posterior neuropil area of the thalamus; *OT* = optic tract; *TT* = tecto-thalamic tract; *Com P* = posterior commissure; 7, 8, 9 = corresponding layers of the optic tectum. The region free of myelinated fibres is the thalamic neuropil, where the majority of tecto-diencephalic fibres terminate. ×40

Figs 4—5. Degenerated fibre terminals in the anterior and posterior thirds of the thalamus on the side of operation. Note that the anterior superior nucleus of the lateral geniculate body (*AN*) and the posterior neuropil of the thalamus (*PN*) are almost free of degeneration. Fink and Heimer technique. ×540

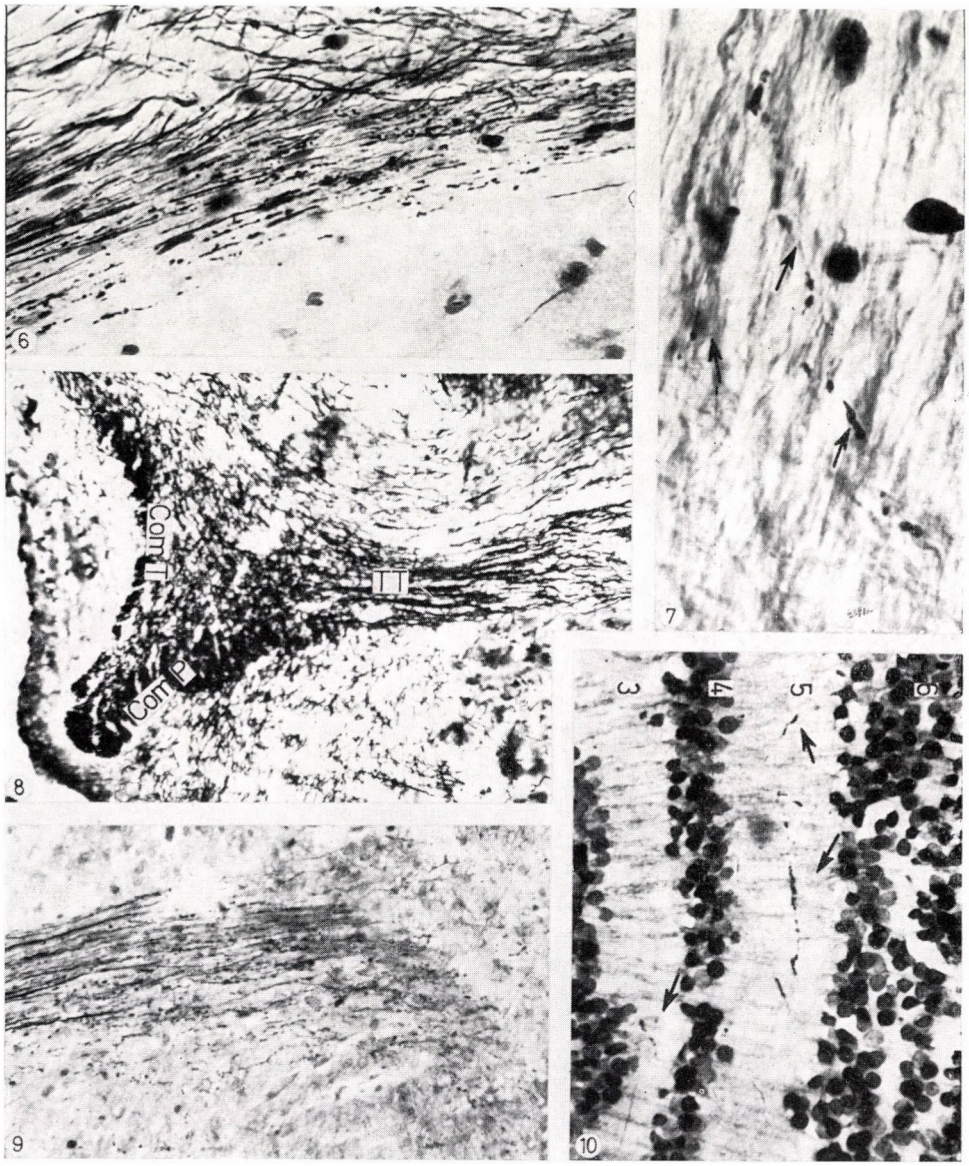

Fig. 6. Horizontal section at the level of the commissura transversa showing large number of degenerated fibres following unilateral tectum ablation. Nauta-Gygax technique. ×270

Fig. 7. Arrows point to three degenerated fibres in the optic nerve on the contralateral side to the operation. Nauta-Gygax technique. ×640

Fig. 8. Sagittal section through the diencephalon and the anterior part of the tectum from a normal frog. Gallyas silver technique. *Com P* = commissura posterior; *Com T* = commissura tecti; *TT* = tecto-thalamic tract. ×80

Fig. 9. Transversal section at the level of the anterior part of the tectum of an operated frog. The picture shows a number of degenerated fibres traversing towards the intact optic tectum (right upper corner) in the pretectal region. Nauta–Gygax technique. ×210

trale) and 9 *(stratum album et griseum superficiale + stratum opticum)* contain nerve cells of diverse forms embedded in a thick meshwork of axonal and dendritic processes. These two layers end at the lateral border of the tectum.

As it was inferred from Golgi studies [14], the efferent neurons of the tectum can be found among the cells of layers 2, 4 and 6. Their axons originate from the shaft dendrites and, on entering layer 7, they turn laterally. Layer 7 collects the efferent fibres and leads them into the basal white matter of the mesencephalon. At the border of the tectum and tegmentum mesencephali these fibres can be seen in vertical orientation, arranged in several thin sheets which are, however, not quite distinctly separated from one another (Fig. 2). In the basal white matter fibres from every alternating sheet turn rostrally, those from the other sheets caudally, forming thus a large rostral and a large caudal projection system. A smaller number of axons of the tectal efferent neurons turn medially in layer 7. These fibres cross over the other tectum and form the *commissura tecti*. In the following a detailed description is given of these three main efferent systems and of the connections they establish with different parts of the brain along their courses, as revealed by degeneration studies following unilateral tectum ablations.

The rostral projection system

A considerable part of the tectal efferent outflow is represented in this system. At leaving the tectum the fibres can be found medially to the lateral bundle of the optic tract, intermingled with the fibres of the caudal projection system (Fig. 16). A little more ventrally the fibres begin to separate from each other, and those belonging to the rostral pathway tend to be located somewhat medially to the others. Fibres originating from the rostral part of the tectum, instead of turning laterally, take a short forward course within the anterior aspect of the tectal hemisphere, then bend medially and join for a short run to the tectal commissure, which they part with a sharp forward turn to form the most dorsal part of the rostral projection system (Figs 3 and 16). These fibres were regarded as a separate pathway and named *tractus tectothalamicus* in the early descriptions. In the diencephalon, adjacent to the optic tract, the system occupies an oval area (in coronal sections) with the long axis in the dorso-ventral direction (Fig. 16). In their further courses the fibres closely follow the optic tract, run around the infundibulum towards the optic chiasm. Right behind the chiasm the fibres converge in a narrow densely-packed bundle and cross over the other side *(commissura transversa* seu *post-*

Fig. 10. Degenerated fibres (arrows) in the 5th and 3rd layers of the optic tectum following contralateral tectum ablation. Numbers from *3* to *6* refer to the corresponding tectal layers. Nauta–Gygax technique. ×337

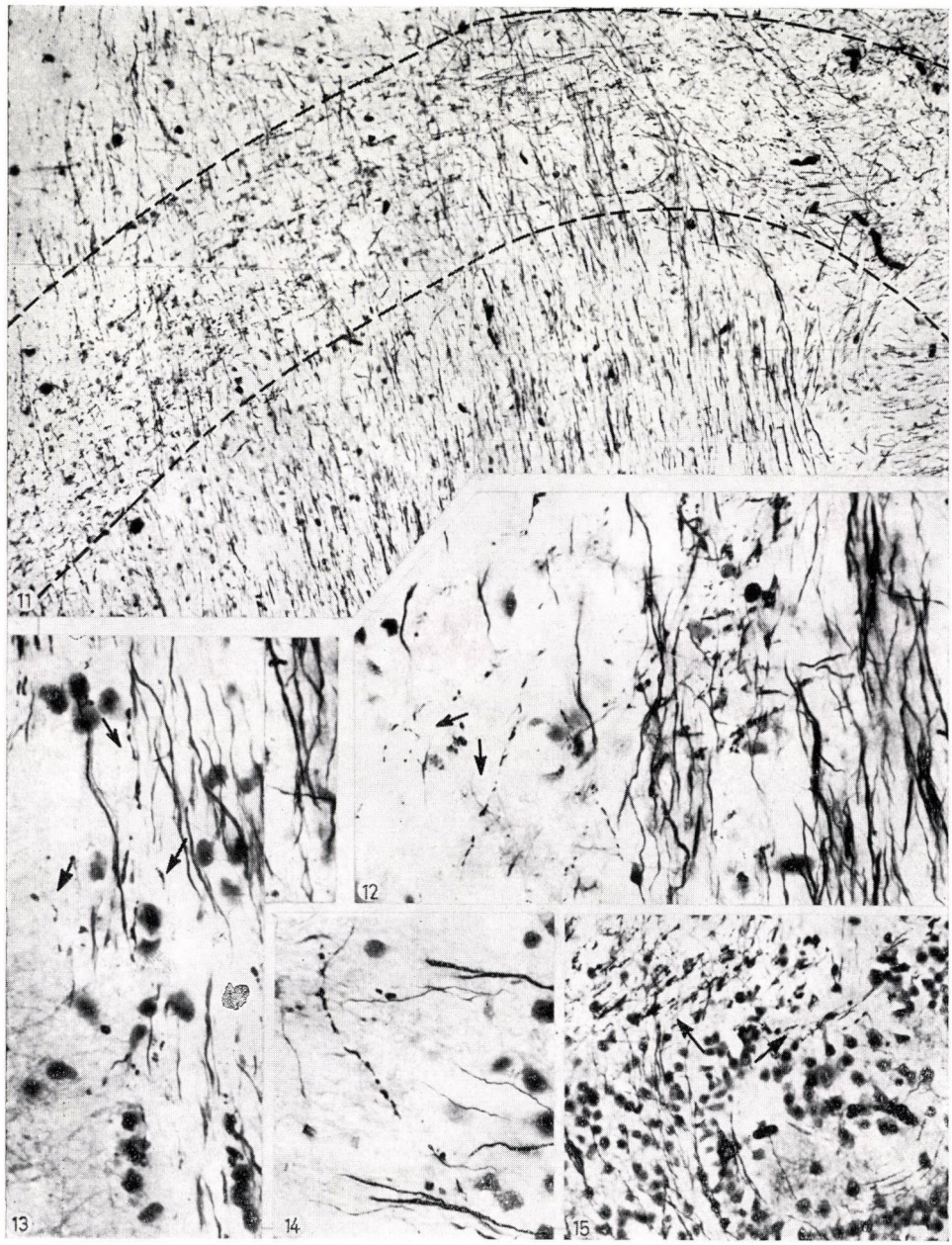

Fig. 11. Photomontage from a horizontal section at the level of the commissura ansulata showing the left half of the basal white matter of the mesencephalon. The right edge of the picture indicates the midline. The left tectum was removed. Parallel lines indicate the commissura ansulata. Nauta–Gygax technique. ×108

Fig. 12. Horizontal section from the medulla oblongata at the level of the tecto-bulbar tract on the side of operation. Arrows point to aberrant degenerated fibres from the lateral bundle entering the gray matter. Nauta–Gygax technique. ×270

optica) (Figs 6 and 16). Lateral to the basal forebrain bundle the crossed fibres take a backward turn, form again a loose tract and can be followed almost as far back as the *nucleus isthmi.* Upon reaching this region the remaining fibres terminate among cells of the *nucleus profundus lateralis.*

This rostral projection system connects the optic tectum to the following parts of the brain.

Tecto-isthmic connections include tectal fibres which terminate in the homolateral *nucleus isthmi* (Fig. 15). According to GAUPP [5] and ARIËNS KAPPERS and co-workers [1], efferent fibres from this nucleus contribute also to the postoptic commissural system, and that is why we have grouped the tecto-isthmic connections among the rostral projections, although the connecting fibres run caudally rather than rostrally. The crossed limb of the postoptic commissure does not reach the contralateral nucleus isthmi.

Tecto-tegmental connections. A number of fibres of the tectal outflow is directly continued into the gray matter of the mesencephalon (Fig. 2). The crossed limb of the postoptic commissure sends also a few fibres to the mesencephalic gray matter. While the uncrossed connections show a preference for spreading in the dorsal part of the tegmentum, the termination of the crossed fibres is confined to the region of the *nucleus profundus lateralis.*

Tecto-diencephalic connections. Both the uncrossed and crossed limbs of the postoptic commissure give origin to several aberrant fibres which terminate in the neuropil of the thalamic nuclei (Fig. 16). On the ipsilateral side nearly the whole neuropil area both of the ventral and dorsal thalamus abounds in degenerated granules, while the degeneration is very rare among the cellular laminae of thalamic nuclei. The accumulation of granules revealing sites of degenerated terminals in Fink and Heimer specimens is most conspicuous in the middle third of the thalamus *(nucleus medius thalami),* and it is somewhat less pronounced in the dorsolateral region of the anterior *(nucleus anterior thalami)* and posterior thirds *(nucleus posterior thalami)* of the thalamus. The habenular region and the hypothalamus are completely void of degeneration. Only very few, presumably traversing degenerated fibres can be seen in the anterior superior nucleus of lateral geniculate body (nucleus Bellonci) and in a round area next to the optic tract in the dorsal neuropil of the posterior thalamus (Figs 3, 4, 5). According to HERRICK [8] this latter corresponds to the primordium of the dorsal nucleus of the lateral geniculate body.

Fig. 13. Horizontal section of the spinal cord at the level of the lumbal segments. Arrows point to degenerated fibres in the ventral part of the lateral funiculus. Nauta–Gygax technique. ×337

Fig. 14. A degenerated fibre enters the dorsal part of the ventral horn at the level of the brachial segments on the side of operation. Nauta–Gygax technique. ×337

Fig. 15. Arrows point to degenerated fibres in the hilus of the nucleus isthmi on the side of operation. Nauta–Gygax technique. ×168

On the contralateral side degenerated terminals can be found only in the neuropil of the *nucleus medius thalami*. The degeneration, however, cannot be traced dorsally as far as on the ipsilateral side.

The transition from the mesencephalon to the diencephalon, which is commonly named *regio pretectalis*, receives its tectal connections mainly through the most dorsal part of the postoptic commissural system (Figs 3, 16). These are fibres which originate in the anterior part of the tectum, and join the tectal commissure. They leave this commissure in a compact bundle and the fibres can be followed forward as far as the medial third of the thalamus (tractus tecto-thalamicus). A few fibres cross over to the other side in the tectal commissure, which is here in close association with the *commissura posterior* (Fig. 16). On the contralateral side degenerated fibres can be found lateral to the *commissura posterior*, terminating in the pretectal region. The regio pretectalis receives thus a stronger ipsilateral and a weaker contralateral innervation from the optic tectum.

Tecto-retinal connections. At the site of decussation in the postoptic region, a few fibres leave the decussating bundle for the chiasm, and enter the contralateral optic nerve (Fig. 7).

The caudal projection system

As the fibres of the rostral and caudal projection system become separated in the dorsal part of the mesencephalic white matter, those belonging to the latter undergo a partial decussation in the basis mesencephali and are continued into the medulla oblongata and spinal cord. After taking a position under the lateral surface of the mesencephalon, the uncrossed fibres run directly caudalwards, and they can be followed through the medulla oblongata into the ventral part of the lateral funiculus in the spinal cord. The greater part of the fibres turn rostro-medially, and in a forward bending arch *(commissura ansulata, commissura tegmenti ventralis)*, cross to the other side among the rootlets of the oculomotor nerve (Fig. 11). This decussation occupies a board region in the basis of the mesencephalon, extending from the *nucleus isthmi* to the posterior edge of the infundibulum. The crossed fibres remain close to the midline in a well defined bundle. The caudal projection system establishes the following connections.

Tractus tecto-bulbaris. During its course through the medulla oblongata, both the crossed and uncrossed limbs give origin to several aberrant fibres. Although the Fink and Heimer technique did not prove to be as effective in the medulla and spinal cord as in the diencephalon to show degenerated terminals, the following sites of termination could be observed. On the ipsilateral side degenerated fibres deviate towards the ventral part of the mesencephalic and rhombencephalic gray substance and get lost among small neurons sur-

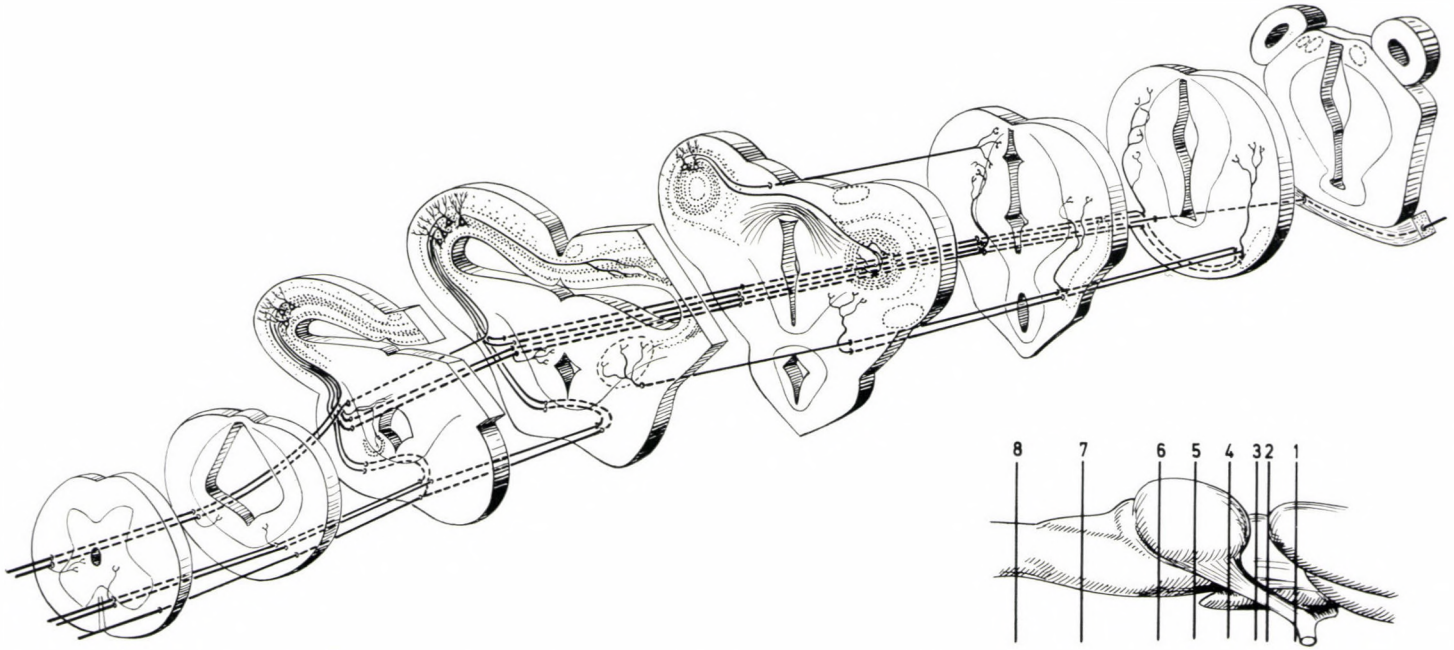

Fig. 16. Schematic representation of the efferent pathways of the optic tectum. Vertical lines in the insert figure indicate the planes of sectioning. In the sections the extent of the gray matter is outlined by thin lines, the tectal stratification is shown in detail. In sections 4 to 6 a few slightly magnified pyramidal neurons indicate the origin of the efferent pathways. The area occupied by the optic tract is indicated by dotted lines in sections 2 to 6. A round area encircled with broken line in section 5 shows the place of the nucleus profundus lateralis, the nucleus isthmi is indicated by dots in section 6. Above the ventricle in section 4 the posterior commissure is represented by solid lines. For further details see text

rounding the motor nuclei of the 5th, 9—10th and 12th cranial nerves (Fig. 12). On the contralateral side the aberrant fibres originate from the crossed ventral bundle, traverse dorsally among the cells of the ventral gray matter and end among the small neurons before reaching the dorsal part.

Tractus tecto-spinalis. After giving rise to several aberrant fibres in the medulla, only a few degenerated fibres reach the spinal cord on the ipsilateral side. They are situated in a tiny bundle occupying a ventral position in the lateral funiculus, and reach down as far as the lumbal segments (Fig. 13). Occasionally one or two degenerated fibres are seen in the zona intermedia and in the ventral horn (Fig. 14).

A well-defined, relatively large bundle descends into the spinal cord on the contralateral side. The degenerated fibres very rapidly decrease in number at the level between the hypoglossal nucleus and the second spinal segment. Within this region many fibres leave the bundle for the intermediate zone of lower medullar and upper spinal gray substance. From the second segment downward the crossed tecto-spinal tract is as modest as on the ipsilateral side, and a few degenerated fibres can occasionally be seen to enter the ventral horn.

Intertectal commissures

A number of tectal efferent axons which turn medially in layer 7 of the tectum subserve intertectal connections. As mentioned above, fibres originating from the anterior aspect of the tectal hemisphere form a solid bundle, which joins the posterior commissure in the pretectal region (Fig. 16). This bundle consists of fibres of three different destinations. A greater part of fibres which soon turn rostrally, establish ipsilateral tecto-diencephalic and tecto-pretectal connections. Another part of the fibres stay in the posterior commissure and end in the contralateral pretectum. These two connections are described above in detail. The rest of the fibres cross to the other side in the roof of the diencephalon and enter the contralateral tectum through layer 7 (Fig. 9). This part of the intertectal commissures is called *commissura tecti diencephali.* In more caudal sections the degenerated fibres gradually become detached from the posterior commissure and from the roof of the diencephalon, and cross to the other side in the midline, where the medial edges of the two tectal hemispheres meet (Fig. 8). These fibres form the *commissura tecti mesencephali.* On reaching the contralateral tectum the degenerated fibres traverse for a while in layer 7, then penetrating cellular layer 6, they continue their course in layer 5 as far as the lateral border of the contralateral tectum (Fig. 10). In layer 5 the fibres arborize, and fine degenerated branches can be followed upward into layer 6 and downward into layers 4, 3 and 2, inclusive.

Discussion

The majority of the results obtained from degeneration studies are in accordance with earlier data found in literature [15, 19]. We could not corroborate the tecto-cerebellar and tecto-habenular connections described earlier [5, 13]. In some cases there are discrepancies between earlier and present observations concerning the origin and destination of the pathways described.

The rostral projection system was regarded as a tegmento-tegmental commissural system connecting the two sides of the mesencephalic tegmentum and torus semicircularis. From degeneration studies it is obvious that, like in reptiles [10, 11], the main source of this pathway is the optic tectum in the frog as well. The pathway connects the tectum to both sides of the diencephalon and of the tegmentum. It is very interesting to compare the sites of termination of the tecto-thalamic fibres with that of the retino-thalamic connections described recently by KNAPP and co-workers [12]. The tectum opticum establishes extensive connections with the middle third of the thalamus on both sides. The anterior and posterior thirds of the thalamus receive fewer fibres and only from the ipsilateral tectum. In addition to their smaller number, the termination of tectal fibres is confined to a dorsolateral area in the anterior, and to a medial area in the posterior third of the thalamus. Areas poor in tectal terminations correspond to the lateral geniculate body and to its nuclei (nucl. Bellonci and the posterior neuropil of the thalamus). Of these nuclei the former receives optic fibres from both eyes, and the latter is supplied by the contralateral eye. Therefore, almost the whole thalamic area is supplied either by direct or indirect optic information, this latter being mediated by the optic tectum. The pretectal region receives both tectal and contralateral optic fibres.

The presence of efferent retinal fibres was first inferred by MATURANA [15], who found unimpaired myelinated fibres in the central stump of the optic nerve 200 days after transection. The present finding shows that a number of these fibres are of tectal origin. The very few degenerated fibres in the optic nerve suggest that the greater number of retinal efferent fibres may, perhaps, originate from the isthmic nucleus, as it has been shown in birds [2, 16, 17].

Of the *caudal projection system* only the tecto-bulbar tract was known and the presence of a tecto-spinal pathway was doubted by several authors [1, 7, 9]. The present finding reveals extensive tecto-bulbar connections in which the ipsilateral connections tend to supply the dorsal half, and the contralateral connections the ventral half of the medulla. We never could see degenerated terminals on, or close to, a motoneuron, presumably the tectal efferent fibres terminate exclusively on internuncial neurons. The rapid decrease of degenerated fibres at the medullo-spinal border on the contralateral side suggests that the majority of tectal impulses reach the spinal cord through intermediate

neurons which are situated in the intermediate gray substance of this region. In view of the fact that the Fink and Heimer technique was not as reliable in the medulla and in the spinal cord as in the diencephalon to show degenerated terminals, further studies are needed to determine the exact sites of termination of the caudal projection pathway.

The intertectal connections were regarded as being two separate bundles: the *commissura tecti diencephali* and *commissura tecti mesencephali*. It is clear from the findings presented that the two commissures belong to the same system, but in the anterior part of the tectum the commissural fibres traverse in the roof of the diencephalon. The functional significance of this system is not known. It seems to be of interest to investigate whether this system establishes connections between identical points of the two tecta.

Addendum

Since the present work was submitted for publication, the abstract of a paper with similar results read by K. RUBINSON in the *Eighty-First Session of the American Association of Anatomists* appeared in *Anat. Rec.*, **160,** 418 (1968).

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EFFERENTE LEITUNGSBAHNEN DES TECTUM OPTICUM BEIM FROSCH

Beim Frosch lassen sich mit der Axondegenerationsmethode drei aus dem Tectum opticum ausgehende Leitsysteme unterscheiden. Die nach vorn gerichtete Leitungsbahn verläuft im basalen Teil des Mesencephalons und Diencephalons, erfährt in der Commissura postoptica eine Überkreuzung und tritt auf die Gegenseite über. Auf der Gegenseite kehrt sie dann zur grauen Masse des Mesencephalons zurück. Die Leitungsbahn stellt die Verbindung mit dem Nucleus isthmii der gleichen Seite, mit dem Tegmentum mesencephali, der Regio pretectalis sowie dem Thalamus auf beiden Seiten und mit der Retina der Gegenseite her. Die nach hinten gerichtete Leitungsbahn ist der Tractus tecto-bulbaris und tectospinalis. Der Großteil der Fasern erfährt in der Commissura ansulata eine Überkreuzung, und sowohl die ungekreuzten als auch die gekreuzten Fasern lassen sich bis zum Lumbalabschnitt des Rückenmarks verfolgen. Im verlängerten Mark gibt die Leitungsbahn Fasern an die die motorischen Kerne der Gehirnnerven umgebende graue Masse ab. An der Grenze des verlängerten Marks und des Rückenmarks nimmt die Zahl der absteigenden Fasern rasch ab, und die verbleibenden Fasern endigen im Rückenmark im Bereich der Vorderhörner und der Zona intermedia. Die dritte Leitungsbahn verbindet die beiden Dächer (Commissura tecti diencephali und mesencephali). Die Fasern dieser Leitungsbahn endigen in der 2.—6. Schicht des Tectum opticum der Gegenseite.

СИСТЕМЫ ЭФФЕРЕНТНЫХ ПРОВОДЯЩИХ ПУТЕЙ ЗРИТЕЛЬНОГО ПОКРОВА У ЛЯГУШКИ

Методом дегенерации аксонов у лягушки можно выявить 3 системы эфферентных проводящих путей, исходящих из зрительного покрова. Направляющийся к переди проводящий путь идет по базальной части среднего мозга и промежуточного мозга, в **commissura postoptica** он тянется перекрещиваясь на другую сторону и возвращается на противоположной стороне к серому веществу среднего мозга. Этот проводящий путь создает связь с **nucleus isthmi** той же стороны, с **tegmentum mesencephali, regio pretectalis** и таламусом на обеих сторонах и со сетчаткой на контралатеральной стороне. Направляющийся к зади проводящий путь — это **tractus tecto-bulbaris** и **tecto-spinalis**. Большая часть проводящих волокон перекрещивается в **commissura ansulata**, причем как перекрещенные, так и неперекрещенные волокна можно проследить до поясничного отдела спинного мозга. В продолговатом мозге эта система отдает волокна к серому веществу, окружающему двигательные ядра мозговых нервов. На границе продолговатого мозга и спинного мозга число нисходящих волокон быстро уменьшается, и остающиеся волокна кончаются в области переднего рога и **zona intermedia**. Третий проводящий путь соединяет два покрова (**commissura tecti diencephali** и **mesencephali**). Его волокна кончаются в 2—6-ом слое зрительного покрова контралатеральной стороны.

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REACTIVITY OF THE SUBCUTANEOUS PITUITARY GRAFT IN CHICKEN EMBRYOS TREATED WITH PROPYLTHIOURACIL

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(Received February 8, 1968)

Synopsis

The development of thiouracil-goitre was investigated by histology, karyometry, ^{131}I-autoradiography and by measuring the thyroid weight, in decapitated chicken embryos bearing subcutaneous pituitary homograft. Goitre formation was in all parameters equal to that observed in thiouracil-treated normal embryos. This shows that deprived of its diencephalic connection the embryonic pituitary gland not only maintains its basal TSH-secreting capacity but it is able to enhance it in response to the enhanced demand.

Introduction

Numerous authors have established that an intact hypothalamo-hypophyseal connection is essential for the maintenance of the normal rate of TSH secretion in the adenohypophysis of mammals [4, 8, 9, 10, 13, 16, 18, 22]. According to ROSENBERG and co-workers [21] these connections are essential also in birds. Disconnection of the hypothalamo-hypophyseal vascular link provokes decreased PB ^{131}I level in the duck, and the iodoprotein (thyroxin, triiodothyronin) production shows similar changes as following hypophysectomy.

In our previous investigations [17], however, we found that the embryonic chicken adenohypophysis grafted under the skin of decapitated recipients has a completely normal TSH-activity, transplanted either before or after the critical period when TSH-secretion starts in the *in situ* pituitaries. Also BETZ [1] found a normal level of TSH, FSH, LH, ACTH and STH production of the transplanted pituitary gland of chicken embryos. These investigations suggest an essential difference between the trophic hormone-secreting potency of the embryonic and of the adult pituitary gland. In contrast to the adult pituitary, the embryonic adenohypophysis deprived of its hypothalamic connections does produce trophic hormones at least at certain basal rate of secretion.

It remained to be elucidated, however, whether the embryonic pituitary graft maintains also its normal reactivity, i.e., whether the TSH-thyroid system developed without any hypothalamic connections is able to respond adequately to loads. To answer this question, we used thiouracil administra-

* Dedicated to the 65th birthday of Professor WALTER HOHLWEG

tion as a specific load of the TSH-thyroid system. Since FLORSHEIM and co-workers [6] concluded that thiouracil treatment of pregnant rats provokes goitre formation in the fetuses by activating the fetal hypothalamo-hypophyseal system, we performed our investigations on decapitated chicken embryos. This model [23] excludes the possible role of the hypothalamus in the development of embryonic thiouracil goitre because the whole prosencephalon and diencephalon are removed at a very early stage of development.

Material and method

Chicken embryos were divided into the following groups:
(A) Intact controls (8 embryos)
(B) Intact embryos, treated with thiouracil (7 embryos)
(C) Decapitated embryos (6 embryos)
(D) Decapitated embryos treated with thiouracil (6 embryos)
(E) Decapitated embryos bearing pituitary homograft (9 embryos)
(F) Decapitated embryos bearing pituitary homograft treated with thiouracil (8 embryos)

Surgery: Chicken embryos were decapitated (Groups C, D, E and F) on the 2nd day of incubation at the mesencephalic level as described by SZÉKELY [23]. One pituitary was transplanted under the skin of the abdominal wall of each decapitated recipient (Groups E and F) on the 6th day of incubation. The pituitary grafts were taken from chicken embryos on the 11th or 12th day of incubation.

Thiouracil administration: Daily propylthiouracil doses of 2 mg were injected into the chorioallantois on the 11th, 12th and 13th days of incubation.

Evaluation: 10 μCi of ^{131}I was given simultaneously with the last propylthiouracil injection. Twenty-four hours later embryos were killed, thyroid glands removed and subjected to autoradiography as described in a previous paper [11].

The density of the autoradiographs was evaluated with the aid of a Zeiss G 11-type fast photometer. A few of the autoradiographs were stained with haematoxylin-eosin for photographic purposes. In some cases one thyroid lobe was fixed in "SUSA" solution, embedded in celloidin-paraffin for karyometric evaluation. Karyometry was performed with the aid of a half-automatic karyometric instrument designed by PALKOVITS and CSAPÓ [19]. Student's "t"-test was used for statistical analysis.

Results

Thyroid weight

As demonstrated in Fig. 1, in normal embryos (Group B) thiouracil treatment caused the same goitre formation as generally seen in adult animals. Decapitation depressed the weight of the thyroid gland (C) and thiouracil administration failed to increase it (D). In decapitated embryos bearing pituitary homograft (E) the increase of the thyroid weight after thiouracil administration (F) was similar to that which occurred in thiouracil-treated intact ones. Thyroids of the decapitated embryos with pituitary homograft (E) were heavier than those of the intact embryos of the same age. This comes from the fact that the pituitaries grafted into 6-day-old embryos were taken from 11- or 12-day-old donors, i.e. the pituitaries transplanted were by 5—6 days older than the

recipient embryos. We observed earlier [17] that the onset and intensity of the TSH-thyroid activity depend first of all on the age of the pituitary gland and not on the developmental stage of the thyroid gland of the recipient.

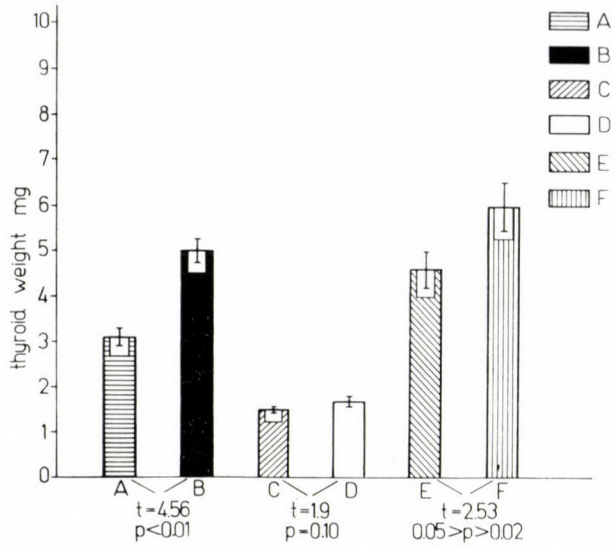

Fig. 1. Thyroid weight (mean value ± standard error) in chicken embryos: normal embryos, decapitated embryos and decapitated embryos bearing pituitary homograft with or without thiouracil treatment. *A* 14-day-old normal; *B* 14-day-old normal + thiouracil treatment; *C* 14-day-old decapitated; *D* 14-day-old decapitated + thiouracil treatment; *E* 14-day-old decapitated bearing pituitary homograft; *F* the same as E + thiouracil treatment

Thyroid histology and karyometric observations

Whereas no histological sign of goitre development was observed in decapitated and goitrogen-treated embryos, thiouracil administration provoked a characteristic picture of thiouracil goitre both in normal and in decapitated embryos bearing pituitary homograft (Fig. 2a, b).

Karyometry is a highly sensitive method for the detection of goitre formation [14, 15]. Nuclear enlargement, characteristic of the acute phase of goitre development, was equally present in both thiouracil-treated groups, i.e., in normal embryos and in decapitated ones bearing pituitary homograft (Fig. 3). Similarly to the histological reaction of the thyroid glands, also the nuclear size spectra of normal controls and of decapitated embryos bearing pituitary homograft were completely in the normal range.

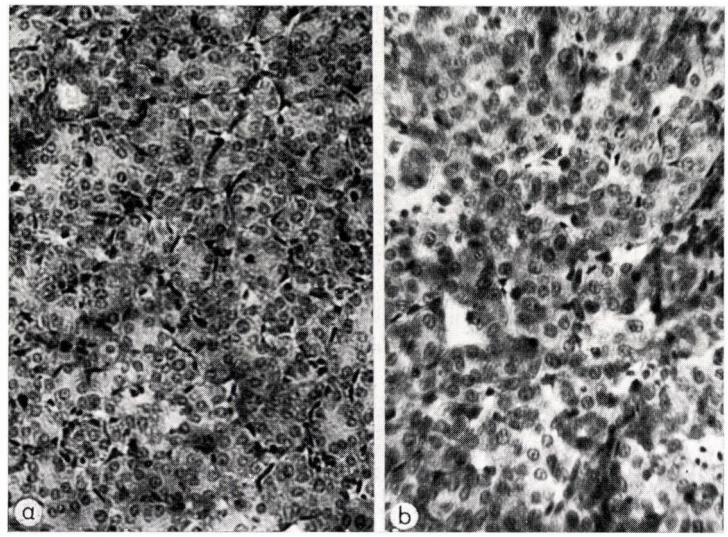

Fig. 2. Effect of thiouracil on the histological character of the thyroid gland of decapitated embryos (a) and of decapitated embryos bearing pituitary homograft (b). Haematoxylin and eosin stain. Magnification ×450

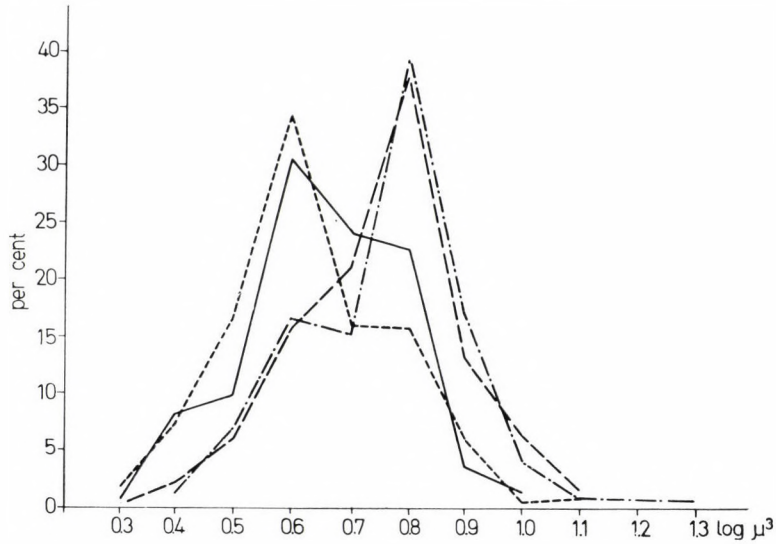

Fig. 3. Nuclear size spectra in the thyroid gland of normal and decapitated chicken embryos bearing pituitary homograft, with or without thiouracil treatment. Each curve represents the mean of 5 individual cases. The logarithms of nuclear volumes (abscissa) are plotted against the percentual occurrence (ordinate) of the given nuclear sizes. Sign. diff.: $t = 9.2$; $p < 0.01$ between the intact controls and thiouracil-treated normal embryos; $t = 16.5$; $p < 0.01$ between the decapitated embryos bearing pituitary homograft, with or without thiouracil treatment. ——— 14-day-old normal; — — 14-day-old normal + thiouracil treatment; – – – – 14-day-old decapitated bearing pituitary homograft; — · — 14-day-old decapitated bearing pituitary homograft + thiouracil treatment

^{131}I-accumulation

The rates of ^{131}I-accumulation for the different experimental groups are summarized in Fig. 4. Both normal and decapitated embryos with subcutaneous pituitary graft showed a fairly high degree of ^{131}I-uptake (Fig. 4A, E; Fig. 5a, c). Thiouracil administration depressed the ^{131}I-uptake of the thyroid

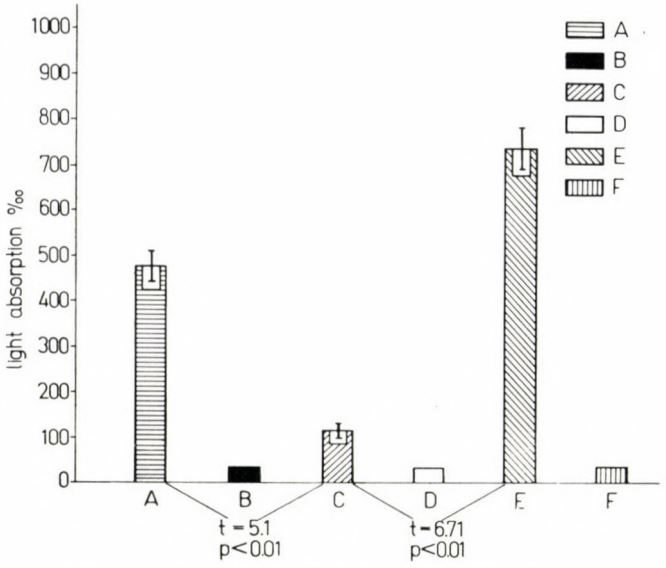

Fig. 4. ^{131}I-accumulation in the thyroid gland (mean value ± standard error) of normal chicken embryos, decapitated embryos and decapitated embryos bearing pituitary homograft, with or without thiouracil administration, measured by quantitative autoradiography. Ordinate: per cent light absorption of the autoradiographs in a Zeiss fast photometer. In Groups B, D and F the activity was unmeasurably low. *A* 14-day-old normal; *B* 14-day-old normal + thiouracil treatment; *C* 14-day-old decapitated; *D* 14-day-old decapitated + thiouracil treatment; *E* 14-day-old decapitated bearing pituitary homograft; *F* the same as E + thiouracil treatment

gland to a level below the sensitivity of the fast photometer in all the thiouracil treated groups (Fig. 4B, D and F; Fig. 5b, d). The primary point of attack of thiouracil inhibiting the incorporation of inorganic iodide in thyronins is in the thyroid gland. The depressed basal activity of the thyroids of thiouracil treated decapitated embryos (Group D) is an expression of this primary effect of thiouracil.

Discussion

Fetal goitre has been observed in rats if in the mother animals the thyrotrophic area of the hypothalamus had been destroyed. On this observation FLORSHEIM and co-workers [6] assumed that fetal goitre might be a consequence of the activation of the fetal hypothalamo-hypophyseal system. It is

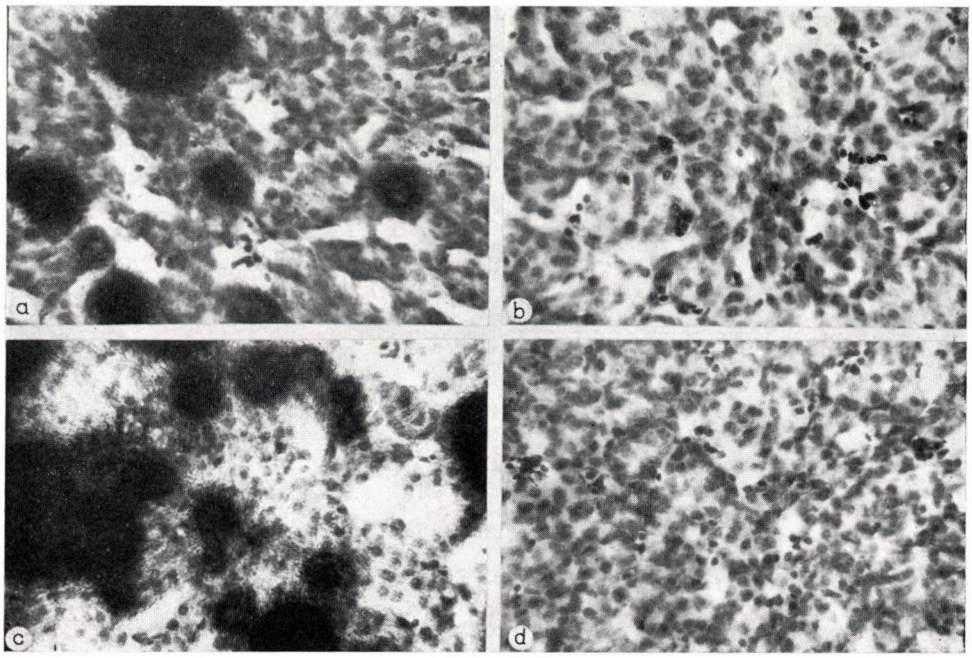

Fig. 5. ^{131}I-autoradiographs of normal and decapitated chicken embryos bearing pituitary homograft with or without thiouracil treatment. All embryos were sacrificed on the 14th day of incubation. The thyroid autoradiographs were stained after with haematoxylin-eosin. Magnification uniformly×450. *a*—Normal embryo. *b*—Normal embryo with thiouracil treatment. No sign of ^{131}I-accumulation. *c*—Decapitated chicken embryo bearing pituitary homograft. *d*—Decapitated embryo bearing pituitary homograft, treated with thiouracil. No sign of ^{131}I-accumulation

known that TSH-regulation is localized in an area of the brain more extended than the basal part of the anterior hypothalamic area [3, 18]. If so, no complete extinction of TRF production can be expected following bilateral lesion of the thyrotrophic area. Therefore, an effect of TRF acting through the general circulation, as suggested by FLORSHEIM and co-workers [5] and KENDALL and co-workers [12], could not be excluded with security.

The present results, obtained on an experimental model excluding the influence of any hypophysiotrophic factors of hypothalamic origin furnished fairly conclusive evidence against the assumption of FLORSHEIM and co-workers [6]. Contrariwise, our results suggest that the embryonic pituitary gland, even completely disconnected from the hypothalamus, is able to secrete a sufficient amount of TSH to induce a thiouracil-goitre. This is the case, however, only with the embryonic pituitary. No goitre formation was observed in thiouracil-treated hypophysectomized rats bearing even 16 adult pituitary glands [12]. A formation of thiouracil goitre could be elicited in hypophysectomized rats exclusively by transplanting pituitary tissue from newborn donors

[20]. These results show a marked difference between embryonic and adult pituitaries concerning their TSH-secreting capacity. Apparently, the embryonic hypophysis has a "full autonomy" in secreting TSH and in increasing its secretory activity under enhanced demand. This autonomy declines successively after birth as suggested by our observation that the subcutaneous embryonic pituitary homografts degranulate and show a series of regressive changes form the 10th day on after hatching.

Portal circulation develops in the rat on the 5th postnatal day [7], and it was shown by CAMPBELL and co-worker [2] that LRF production starts in the hypothalamus at the same time when the primary plexuses of the portal circulation are developed. Peculiar is the coincidence of the development of the hypothalamo-hypophyseal portal circulation with the successive development of the hypothalamic control over the pituitary trophic hormone secretion and with the simultaneous regression of the embryonic autonomy of the adeno-hypophyseal functions. The physiological role and nature of this coincidence need further experimental elucidation.

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REAKTIONSFÄHIGKEIT DES SUBKUTANEN HYPOPHYSENVORDERLAPPEN-TRANSPLANTATES IN PROPYLTHIOURACIL BEHANDELTEN HÜHNER-EMBRYONEN

Verfasser untersuchten die Thiouracilkropfbildung in normalen, in dekapitierten, sowie in dekapitierten Hühnerembryonen mit subkutanem Hypophysenvorderlappentransplantat. Schilddrüsengewichtserhöhung, histologisches Bild, kernvariationsstatistische Verschiebungen und ^{131}I-autoradiographische Veränderungen dienten als charakteristische Zeichen der Schilddrüsenaktivität. Eine Entwicklung von Thiouracil-Struma war gleichartig nachweisbar in Thiouracil behandelten normalen, sowie in dekapitierten Embryonen mit Hypophysenvorderlappentransplantat. Keine Kropfbildung war aber in Thiouracil behandelten einfach dekapitierten Embryonen zu bemerken. Die vorliegenden Angaben weisen darauf hin, daß die embryonale Hypophyse, ohne hypothalamische Verbindungen auch ihre normale Reaktivität, nicht nur die Basalsekretion behalten hat.

ИССЛЕДОВАНИЕ РЕАКТИВНОЙ СПОСОБНОСТИ ПЕРЕСАЖЕННОЙ ПОД КОЖУ ПЕРЕДНЕЙ ДОЛИ ГИПОФИЗА У ЗАРОДЫШЕЙ ЦЫПЛЯТ, ОБРАБОТАННЫХ ПРОПИЛТИОУРАЦИЛОМ

Авторы изучали образование зоба от тиоурацила у нормальных и обезглавленных зародышей цыплят, а также у обезглавленных зародышей цыплят с пересаженным гипофизом. В целях определения функциональной активности щитовидной железы были проведены измерения повышения веса щитовидной железы, исследования гистологической картины, статистические измерения варьирования ядер и авторадиографические исследования с помощью ^{131}J. Тиоурациловый зоб развивается в одинаковой мере как у нормальных зародышей, так и у подобно обработанных обезглавленных зародышей и у зародышей с пересаженным гипофизом. Однако, развитие зоба отсутствует в группе просто обезглавленных и обработанных тиоурацилом зародышей. Эти экспериментальные данные указывают на то, что зародышевый гипофиз и при отсутствии связи с гипоталамусом не только сохраняет свою основную секрецию тиреотропного гормона, а сохраняет также свою реактивную способность в отношении раздражений со стороны внешнего мира.

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ACCESSORY PARATHYROID TISSUE IN THE RAT THYMUS

ELECTRON- AND LIGHT-MICROSCOPIC AUTORADIOGRAPHIC STUDIES

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Synopsis

Accessory parathyroid, situated in the interlobular connective tissue of the rat thymus, has the same ultrastructure as the normal parathyroid. It contains two types of cells: dark active cells and light, inactive or aged forms. Light-microscopic autoradiography showed the aminoacid uptake of the accessory parathyroids to be the same as that of the corresponding normal organs.

Introduction

In the interlobular connective tissue of the thymus numerous authors [1, 2, 3, 4, 7, 8, 11, 14] have found by light microscopy a structure, which consisted of epithelial cells and proved to be parathyroid. It is presumably due to such accessory parathyroids that parathyreoidectomy is not followed by deficiency symptoms in certain mammals [3, 8, 11]. The present experiments were designed to determine the electronmicroscopic structure of the accessory parathyroids encountered in the thymus, and to ascertain their functional significance on the evidence of autoradiograms examined under the light microscope.

Material and method

Randomized adult female Wistar rats were used.

Electron microscopy. In animals in ether narcosis the thorax was opened, the apex of the heart cut off, and a cannula was inserted in the aorta through the left ventricle. The blood was then washed off through the tube with Plasmodex HUMAN* (a 6% solution of dextran in saline). Perfused at 37° C for 15 min with 4.5% glutaraldehyde buffered by 0.1 *M* phosphate (pH 7.2), the thymus was then removed, cut into pieces and fixed for two hours in the same solution. After one-hour postfixation with osmium tetroxide, the material was dehydrated in alcohol, embedded in araldite, counterstained with a 50% solution of uranylacetate in methanol and then examined in an electron microscope, type JEM 6C. The half-thick sections were stained for three minutes with a warm solution of toluidine blue.

Light microscopic autoradiography. The animals were given 100 μCi, and 24 hrs later 50 μCi aqueous solution of leucine-^{3}H and phenylalanine-^{3}H of 1 : 1 activity. (Specific activity of leucine 5.38 Ci/*mM*, that of phenylalanine 1.03 Ci/*mM*.) The animals were sacrificed 24 hrs after the second administration of the isotope. The thymus and parathyroids were fixed in Carnoy's fluid and embedded in paraffin. The sections, 6μ in thickness, were mounted with Kodak AR-10 stripping film, exposed for 20 days, developed in Kodak D-19 for five minutes, fixed in Kodak-acid fixative and post-stained in Giemsa solution.

* Institute for Serobacterial Production and Research "Human", Budapest.

Results

Accessory parathyroid tissue was found to be scattered over the interlobular connective tissue of the thymus in the form of islets consisting of epithelial cells (Figs 1, 2). Presumably these accessory parathyroids are supplied with blood from the vessels that supply the thymus. In general, the

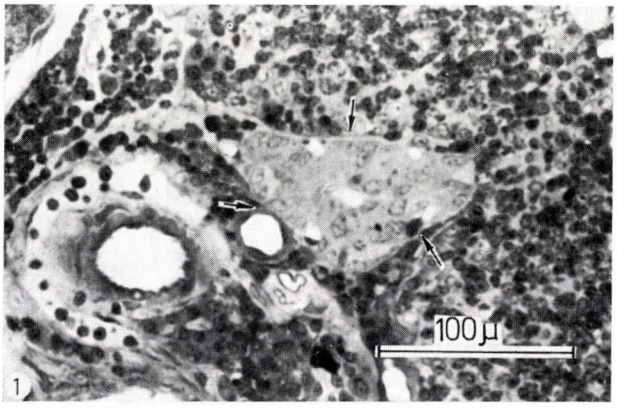


Fig. 1. Toluidine blue stained half-thick section. Arrows point to accessory interlobular parathyroids well distinguishable from the tissue of the thymus. Note capillaries in the centre and at the boundary of the cell group

vessels are situated at the boundary of the islets, but capillaries are sometimes in their interior with parathyroid cells radially arranged around them (Fig. 3). The vessels are lined by endothelial cells showing typical fenestration. The endothelial cells are separated from the subendothelial space, varying in width, by a basement membrane 200—300 Å in thickness. This separates the islets from the subendothelial space and the surrounding connective tissue (Figs 2, 4a, b). The epithelial cells of the islets are bridged by zonulae occludentes, zonulae adherentes and desmosomes (Figs 4b, 5, 9).

According to the electron density of the hyaloplasm, light (Fig. 6) and dark cells (Fig. 7) can be distinguished in the epithelial islets. Besides there are numerous cells of medium density, transitory forms between the light and dark types.

The nuclei of both types (Figs 6, 7) are irregular in shape, often lobate, with a loose chromatin mass occasionally forming small clumps at the inner side of the nuclear membrane.

Higher electron density and abundance of ribosomes characterize the dark cells (Figs 7, 8). Ribosomes are scattered in the cytoplasm, some being attached to the membranes. Cisternae of the ergastoplasm, and mitochondria are generally arranged around the moderately developed Golgi apparatus

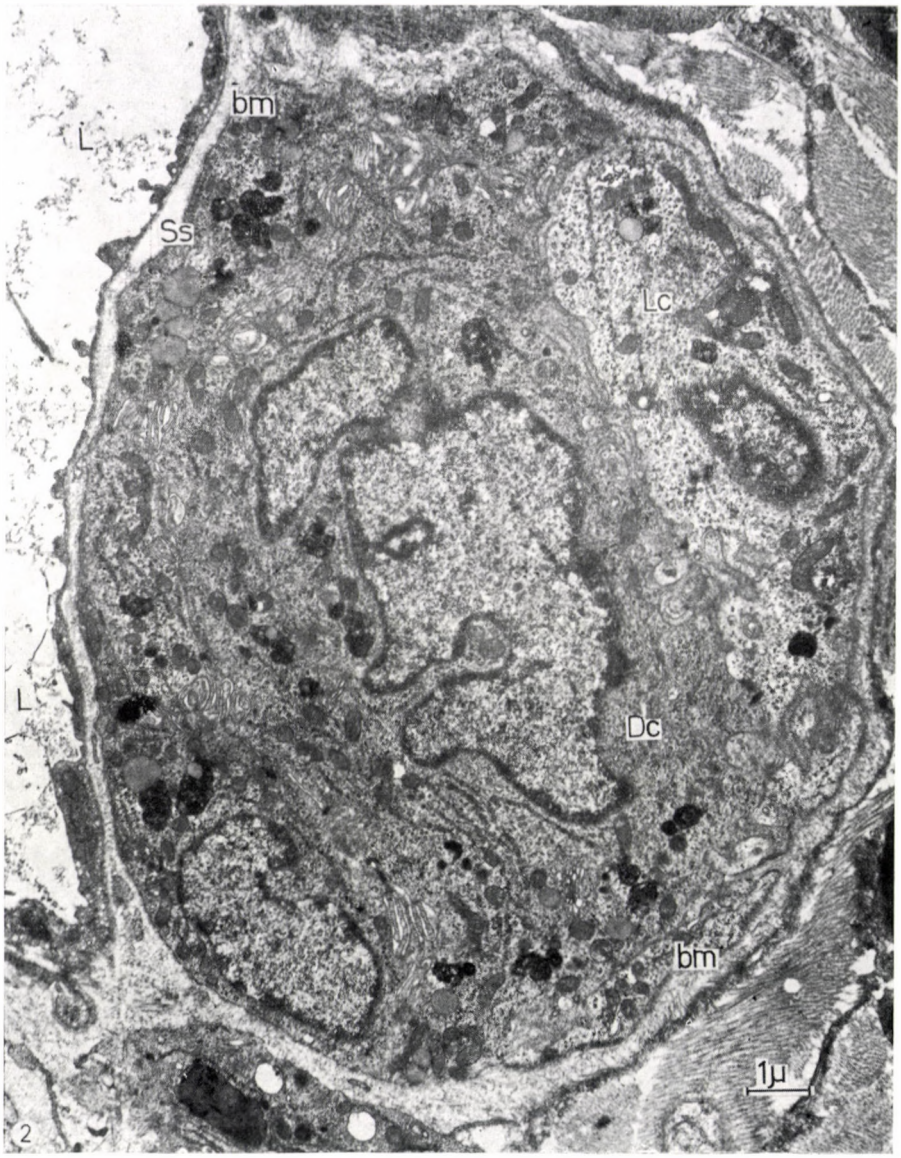


Fig. 2. Survey picture of the parathyroid. It is surrounded by connective tissue. Endothel-lined vessel is lying against the surface of the cell group
Dark (Dc) and light (Lc) cells are clearly distinguishable in the parathyroid tissue. *L* = lumen of vessel; *bm* = basement membrane; *Ss* = subendothelial space

having cisternae dilated at some sites and vesicles containing a substance of medium density.

It is the low electron density of the hyaloplasm, that makes light cells (Figs 6, 9) conspicuously different from the dark ones. Besides, the light cells

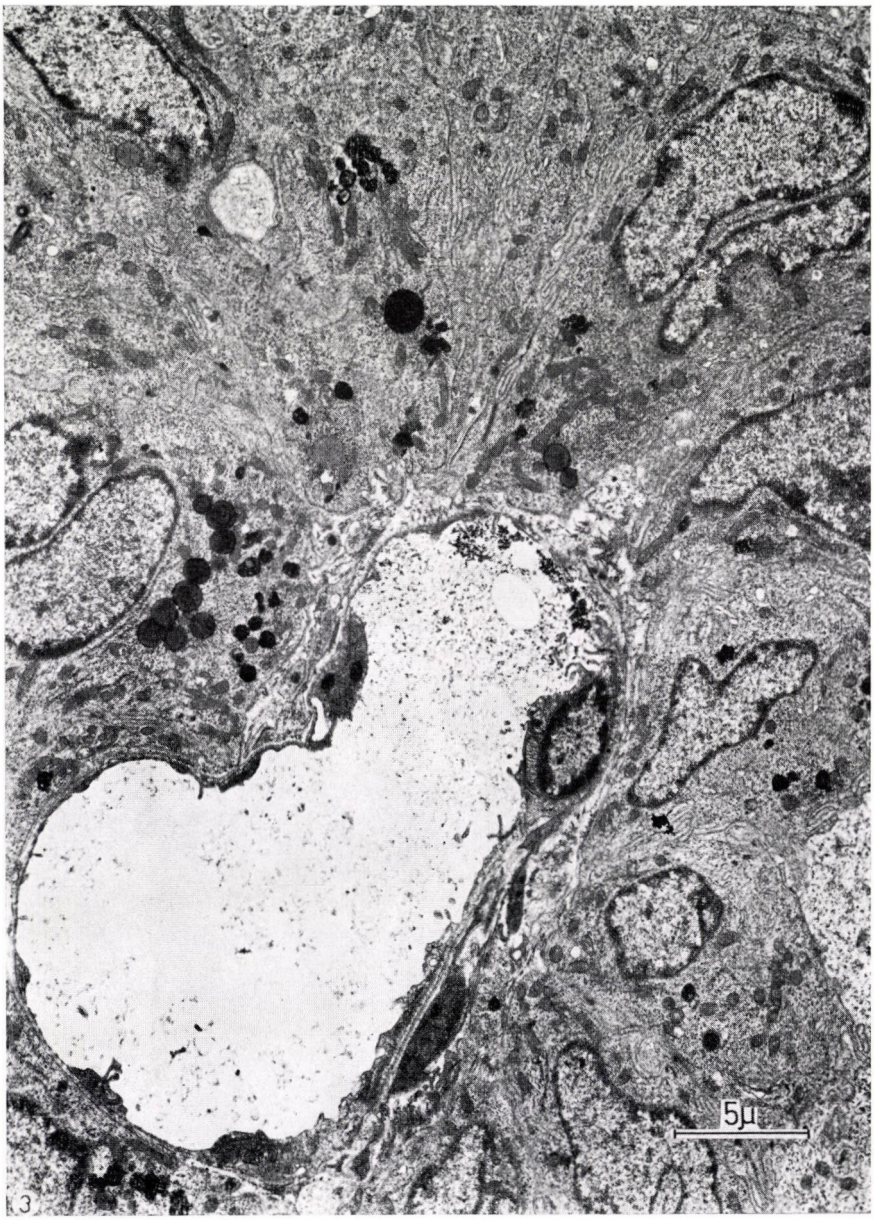


Fig. 3. Radial arrangement of cells around capillary, which coated with fenestrated endothelium, is running in the interior of the accessory parathyroid

are considerably poorer in free ribosomes and ergastoplasmic cisternae, and their Golgi apparatus is less developed. The complex of ergastoplasm – Golgi apparatus – mitochondria, characteristic of the dark cells, was not seen in the light ones.

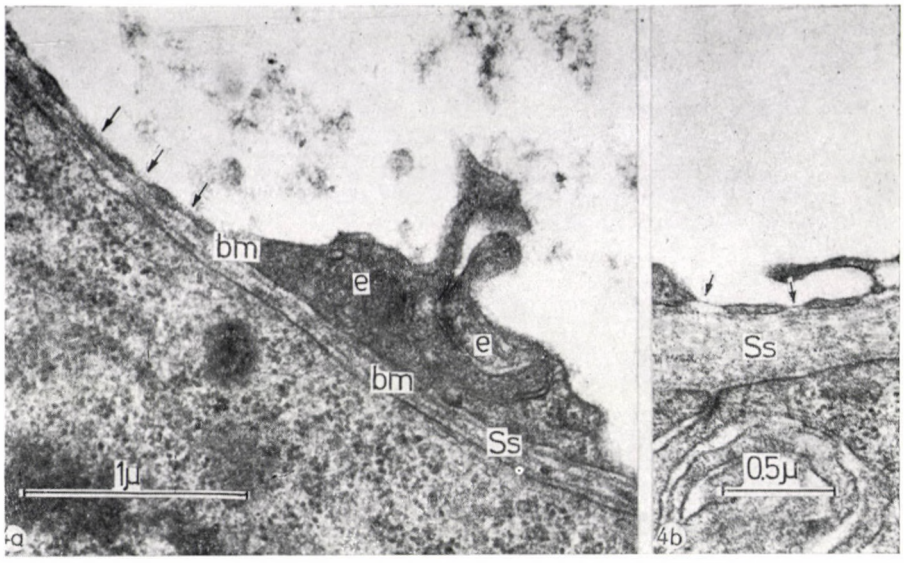


Fig. 4a—b. Barrier between the parathyroid cells and the vessel lumen consists of the basement membrane (*bm*) of the epithelial and endothelial cells, the subendothelial space (*Ss*) of varying width and the fenestrated endothelium (*e*) of the capillary (arrows). The cells of the latter are connected by various structures where microvilli are frequently seen. The intercellular space between the parathyroid cells is closed by macula adherens

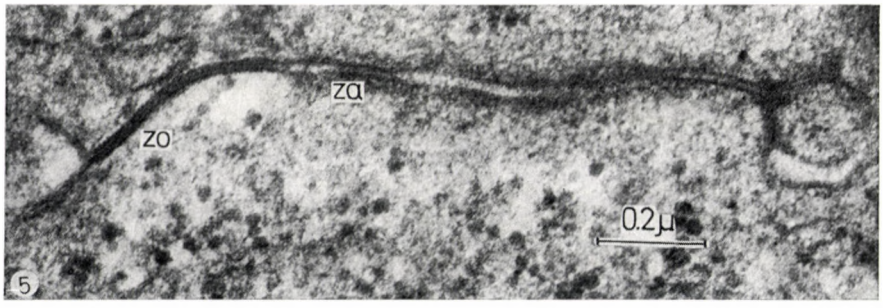


Fig. 5. Cells of the gland are bridged by zonulae occludentes (*zo*) and zonulae adherentes (*za*)

Both types contain a few membrane-bounded granules of variable density and size. No topographical connection seems to exist between these granules and the Golgi bodies. Homogeneous lipid droplets of low density occur in dark and light cells alike. The latter type contains more frequently large amounts of lipofuscin-like pigment, possibly evidence of reduced activity.

Light microscopic autoradiographic investigations revealed a cell group in the interlobular connective tissue of the thymus taking up a considerable amount of labelled leucine and phenylalanine. The light-microscopic struc-

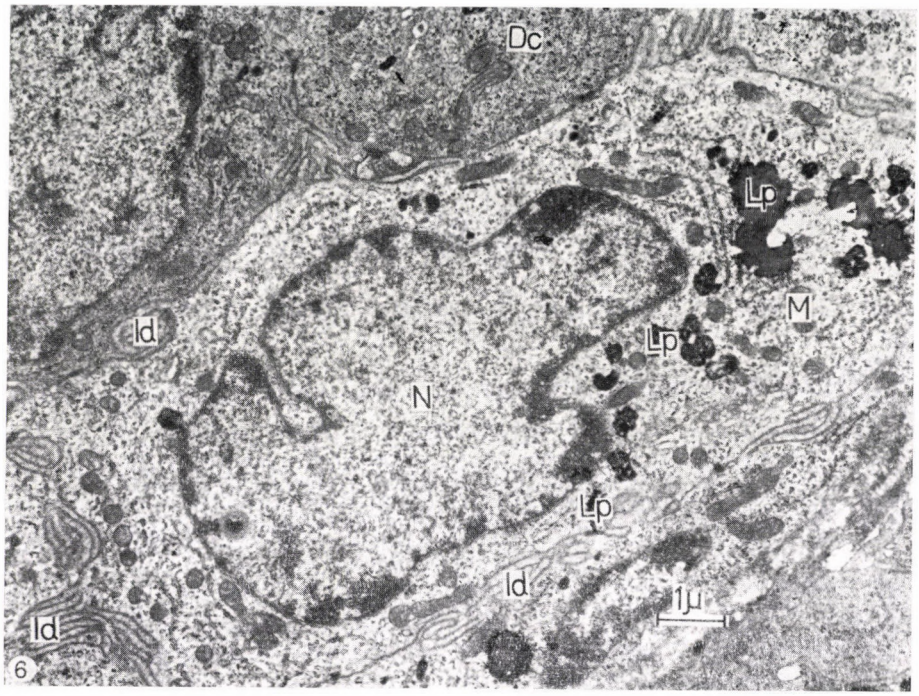


Fig. 6. Survey picture of light cell. The nucleus (*N*) has an irregular shape and a loose chromatin substance; small clumps of chromatin are attached to the nuclear membrane. The hyaloplasm of low electron density contains occasional scattered mitochondria (*M*) and much lipofuscin pigment (*Lp*). It is connected to the adjacent cells by intricate interdigitation (Id). *Dc* = dark cell

ture of the cell group pointed to its parathyroid nature. Namely, among all amino acids it is leucine and phenylalanine which are percentually dominant in the parathyroid [9]. These two amino acids are therefore suitable for the determination of parathyroid activity. They are readily incorporated by normal parathyroids, and the granules occupy usually a perinuclear position, although they sometimes show a diffuse arrangement above the cell (Fig. 10a). Incorporation of these amino acids is similarly intensive over the area of the accessory parathyroids in the thymus, but the arrangement of the granules is mostly diffuse (Fig. 10b). The fact that normal and accessory parathyroids are equally ready to take up the two amino acids shows the latter to have functional potency.

Discussion

The epithelial nature of the cells grouped in the interlobular connective tissue of the thymus, their arrangement in foci and bundles, and the large number of capillaries connected with them point to their endocrine character.

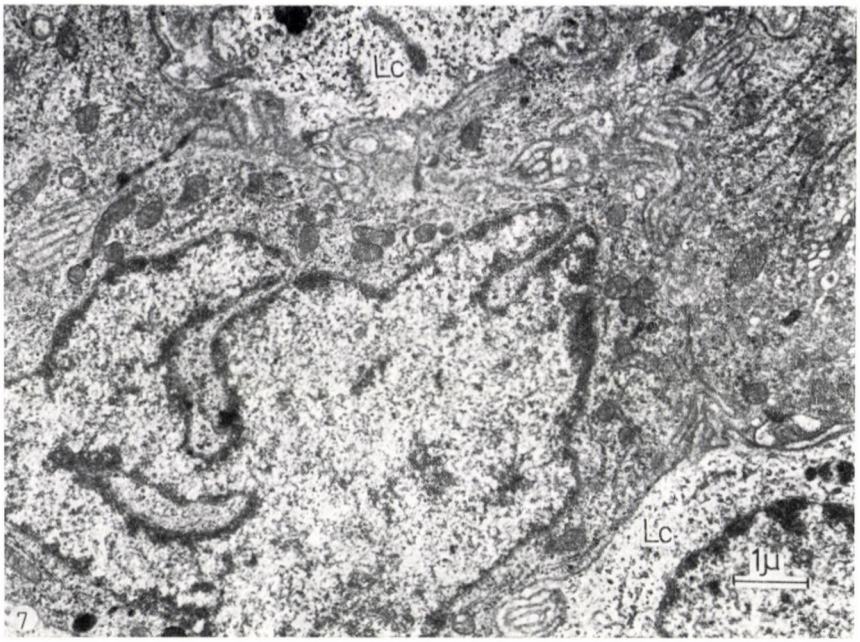


Fig. 7. Survey picture of a dark cell. The lobate nucleus contains a loose chromatin substance. Note the high electron density of the hyaloplasm

The regular fenestration of the endothelial cells of the capillaries on their surface towards the epithelial cells is a further proof in favour of their endocrine character. The thyroid and parathyroid glands are those two endocrine organs which originate from the same anlage as the thymus, i.e., the pharyngeal entoderm. Phylogenetic considerations as well as literary data [1, 2, 3, 4, 8, 11, 12, 13, 14] suggest that the islets in question represent one of these two glands. Results of both light- and electron-microscopic studies have verified their parathyroid nature.

The rat parathyroid contains, according to literature, only a single type of cells. The cells of high, and those of low electron density represent different functional phases of one and the same type [5, 6, 15]. We observed light and dark cells in the accessory parathyroid tissue, and found that the dark cells to be seen the active form. The activity of the dark cells has been confirmed by Roth and Raisz [10]. These authors observed that in rat parathyroid organ cultures growing in a medium deficient in calcium, the dark cells increased in number as a sign of hypersecretion. The functional potency of the dark cells was indicated in the present experiments by the close topographical connection of the Golgi apparatus, the ergastoplasm and the mitochondria, as well as the abundance of ribosomes. The absence of this characteristic functional complex

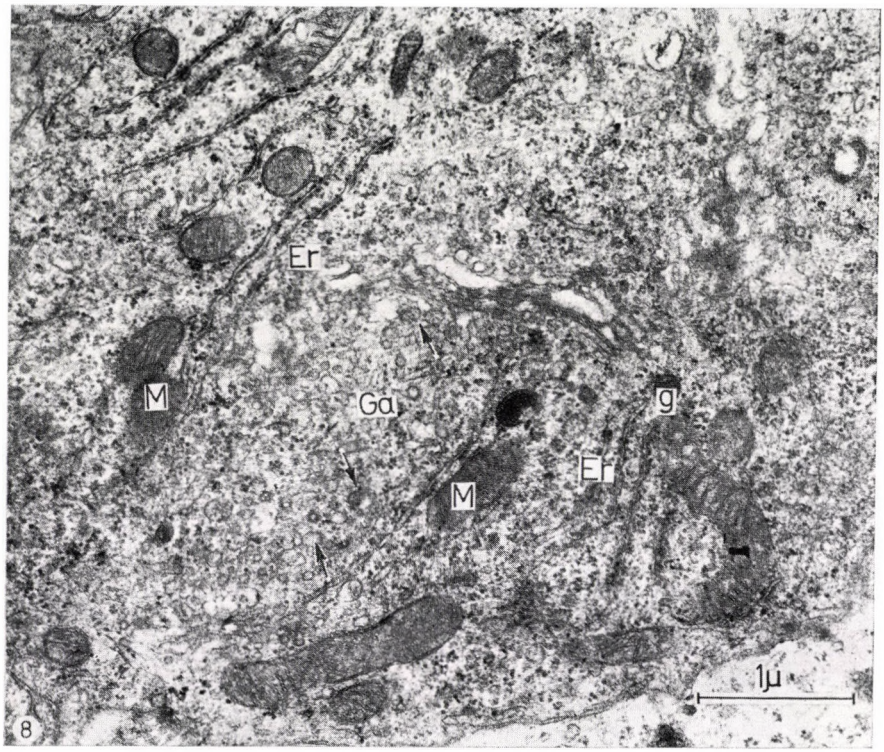


Fig. 8. The complex consisting of ergastoplasm (*Er*,) Golgi apparatus (*Ga*) and mitochondria (*M*) is a characteristic feature of the cytoplasm of dark cells. The cisterns of the Golgi zone are slightly dilated at some sites, its vesicles contain moderately electron-dense substance (arrows). Also occasional membrane-bounded electron-dense granules (*g*) appear in the cytoplasm

and the presence of large amounts of lipofuscin-like pigment in the light cells suggest that these are quiescent, inactive or senile cells. It is, on the strength of these morphological properties, safe to assume that the dark cells actually produce hormone and in this way support the activity of the normal parathyroids. This assumption is supported by the incorporation of labelled amino acids in the accessory parathyroids, a phenomenon indicative of their ability to synthetize protein.

The next problem was to decide whether the dark cells were identical with the cells that incorporate labelled amino acids. It is probable that also the transitory forms belong to the active type, since the typically light cells were quite as much in minority in the electron-microscopic preparation as were those cells in the light-microscopic picture which failed to take up amino acids. The present experiments show therefore the two kinds to be identical, although more reliable identification requires electron-microscopic autoradiographic investigations.

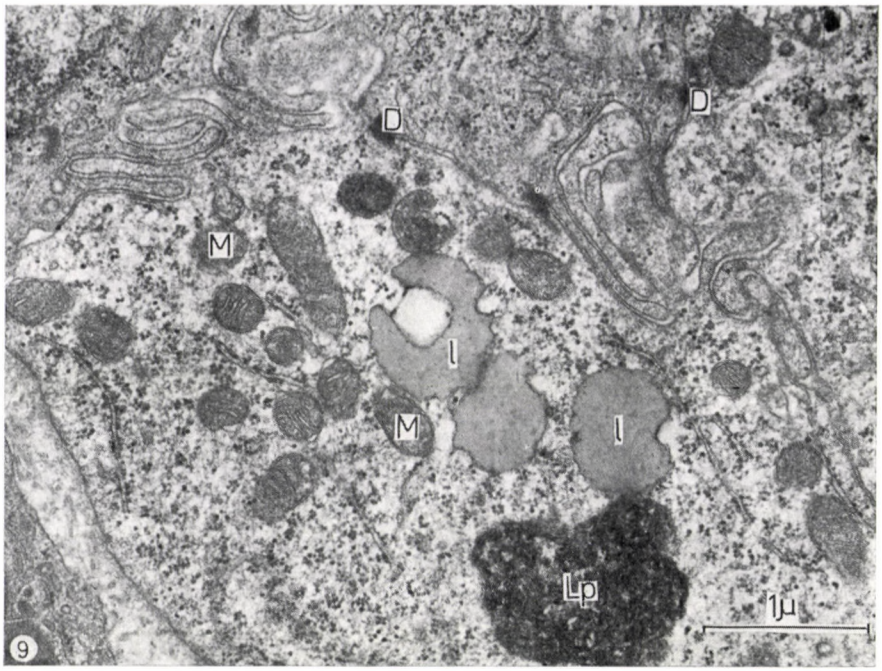
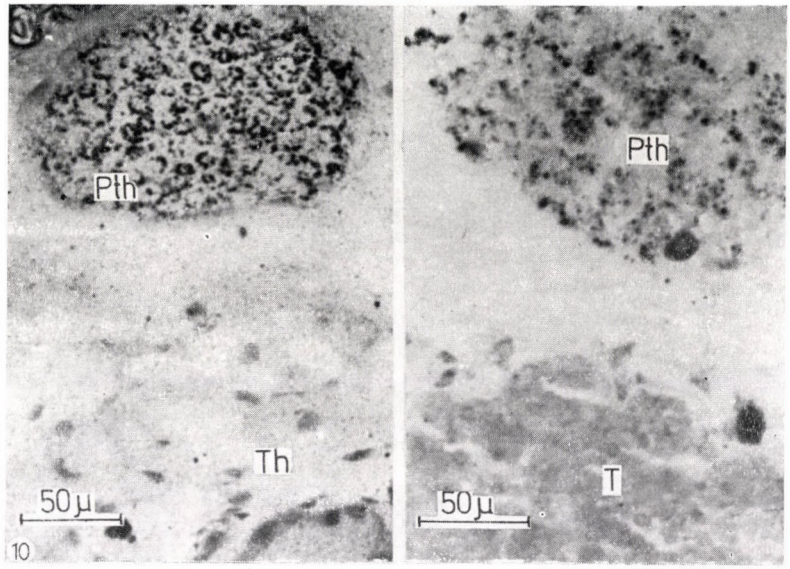


Fig. 9. Cytoplasm of light cell. Free ribosomes and a few mitochondria (*M*) are visible in the hyaloplasm of the low electron density. The cell contains as constant structures lipid droplets (*l*) and lipofuscin granules (*Lp*) composed of elements of different electron densities. Connection with the adjacent cells by interdigitation and desmosomes (*D*) are clearly distinguishable

The accessory parathyroids seem to be functionally active as shown by their fine structure and on the evidence of light-microscopic autoradiography. Considering that the sections originated from normal animals, it can be presumed that accessory parathyroids produce hormone even under physiological conditions supplementing the hormone production by the normal glands. Earlier authors [3, 8, 11] believed that accessory parathyroids start producing hormone only after the normal ones have ceased functioning.

The location of the secretion in the secretory cells is the following question. Granules variable in size and shape, surrounded by membranes of medium electron density were present in both types, although their number was small and they appeared in few cells. No morphological features have proved that these granules were of the true secretory type. Their possible lysosomal nature requires further histochemical investigations. There is still another possibility as regards the nature of the granules. The high electron density of the hyaloplasm in the dark, active cells may mean that the protein-like product of the cells exists in the cytoplasm in a soluble and not in a granular form.The granules would in this case represent condensation products of the soluble protein



a b

Fig. 10. Light-microscopic picture of autoradiogram showing incorporation of labelled amino acid by parathyroid. *a* = Normal parathyroid: *Th* = thyroid; *Pth* = parathyroid. Note perinuclear and diffuse arrangement of granules. Thyroid and background are practically free of granules. (Giemsa's stain.) *b* = Accessory parathyroid (Pth). A minor part of the cells do not take up labelled amino acids. Cortex of the thymus (*T*) and background are practically free from granules. (Giemsa's stain.)

substance. These considerations are, however, outside the scope of this study as they lead to histophysiological problems connected with parathyroid hormone production.

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AKZESSORISCHE NEBENSCHILDDRÜSEN IM THYMUS VON RATTEN

Elektronenmikroskopische und lichtmikroskopische autoradiographische Beobachtungen

Die elektronenmikroskopische Struktur der akzessorischen Nebenschilddrüsen im interlobären Bindegewebe der Rattenthymus ist mit der normalen Nebenschilddrüsenstruktur identisch. Es lassen sich zwei Zellentypen unterscheiden: ein dunkler, auf Grund der elektronenmikroskopischen Struktur aktiver, und ein heller, inaktiver oder veralterter Zellentyp. Die lichtmikroskopische autoradiographische Untersuchung hatte bestätigt, daß die Aminosäurenaufnahme dieser akzessorischen Nebenschilddrüsen mit der Aminosäureninkorporation der normalen Nebenschilddrüsen gleich ist.

ДОБАВОЧНЫЕ ПАРАЩИТОВИДНЫЕ ЖЕЛЕЗЫ В ЗОБНОЙ ЖЕЛЕЗЕ КРЫСЫ

Электронномикроскопические и светово-микроскопические авторадиографические исследования

Электронномикроскопическая структура добавочных околощитовидных желез, локализированных в междольковой соединительной ткани зобной железы крысы, соответствует структуре нормальных паращитовидных желез: Можно выделить два типа клеток: один темный, по своей электронномикроскопической структуре активный тип, и один светлый, неактивный или пристарелый тип клеток. Авторадиографические исследования под световым микроскопом выявили, что поглощение аминокислот этими добавочными паращитовидными железами такое же, как и инкорпорация аминокислот в нормальные паращитовидные железы.

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Acta biol. Acad. Sci. hung., **20**(2) 205—210 (1969)

MECHANISM OF THE FORMATION OF MAST-CELL GRANULES

II. CELL-FREE MODEL

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(Received March 19, 1968)

Synopsis

The formation of mast-cell granules has been studied in a cell-free model. Typical metachromatic granules were formed only if in the model the relative concentrations of the four components of mast-cell granules were approximately the same as in the mast-cell granules themselves. Presence of an alcian-blue-positive component in the granules a phenomenon characteristic of young forms of mast-cells indicates a preponderance of biogenic amines, whereas the safranin-positivity of the mature granules appears to indicate the preponderance of heparin.

Introduction

We have demonstrated [6] that typical metachromatic granules appear in cultured thymic cells if the constituents of the mast-cell granules are contained in the cultured medium. If, among the constituents, only heparin was taken up, the corresponding electron microscopic structure appeared to be an empty vacuole. On the other hand, typical mast-cell granules developed after the uptake of the non-metachromatic constituents histamine and serotonin. It was therefore assumed that histamine and serotonin either took up heparin from the culture medium or induced intracellular heparin synthesis. Considering that from tissue culture models neither the disturbing effect of the components of the medium nor that of cellular activity can be eliminated, we developed a cell-free model for studying the formation of mast-cell granules. The experiments were expected to shed light on the following questions.

(1) Are the components of mast-cell granules able to form bonds and develop granule-like structures in the absence of cells?

(2) Is such formation of bonds influenced by the interproportion of the components?

Material and method

Heparin [12] and histamine [8, 12] were the first two substances known to be components of mast-cell granules. The mast-cell granules of rats and mice contain also serotonin [3, 4, 8, 15], and recent investigations [1, 15] have demonstrated in certain species the presence of dopamin. Accordingly, solutions containing different concentrations of these sub-

stances* were used in the present experiments. In each series of experiment, the concentration of one of the substances was varied, that of the others remained constant. The concentration of heparin and histamine was raised in steps of 1 mg from zero to 9 mg, and that of serotonin and DOPA (the amount of which is considerably less [9, 15] in the mast-cell granule) in steps of 0.1 mg from zero to 0.9 mg. Each component was dissolved in distilled water, and the solutions were dropped into Wassermann tubes kept in a water bath of 37° C in the following order: heparin, histamine, serotonin, DOPA. Then 0.2 ml of 12% gelatin, dissolved in distilled water, was added to each of the mixtures, which were then shaken and left in the water bath for 20 minutes while a macroscopic precipitate was formed in some of the tubes. A smear was prepared from the content of each tube on glass slides, which had been kept for 24 hours in chromosulphuric acid, then thoroughly washed and degreased. The smears were fixed in a 4% solution of formalin for 24 hours, washed with tap water and stained with iron alum-alcian blue (pH 1.02) or iron alum-alcian blue-safranin (pH 1.42) [2] or with the PAS method.

The iron alum–alcian blue–safranin staining was performed as follows: 110 ml of Walpole buffer (pH 1.42) consisting of 60 ml *N* HCl + 50 ml *N* Na acetate was diluted to 250 ml by distilled water; 0.36 g of alcian blue, 0.18 g of safranin and 0.48 g of iron-alum were then dissolved in 100 ml of the diluted buffer; the preparation was then stained with this solution for 10 to 20 minutes. After dehydration (by tertiary butyl alcohol) and clarification, the slides were mounted with Canada balsam.

Results

Addition of the different granule components and gelatin caused precipitation in those tubes in which the interproportion of the components was approximately the same as in mast-cell granules, while no precipitation occurred in tubes with heparin excess, or in which the amount of histamine surpassed that of heparin. Excess serotonin or DOPA had no effect in this respect. Detailed results are shown in Table 1.

No precipitation took place in the absence of gelatin.

Staining with toluidine blue resulted in the appearance of metachromatic grain-like particles in all cases of precipitation. Their size was many times larger than that of mast-cell granules; they had diameters of about 9 to 10 μ, and their interior appeared to be inhomogeneous. Smears from tubes without macroscopic precipitation contained no such particles. With alcian blue—safranin, the grains stained blue (Fig. 1) or red (Fig. 2), while certain specimens of the series displayed both blue and red colours. In Table 1 the compositions resulting in metachromatic granules are enframed. When the respective concentrations were 3, 1, 0.1, and 0.1, the precipitation was exceptional: in addition to red granules, a fine red precipitation was observed (Fig. 3).

Alcian blue without safranin failed to stain the safranin-positive granules, these appeared as unstained areas in a stained background (Fig. 4).

Discussion

We developed in the present experiments a model of the genesis of mast-cell granules. In the model only the most important components (heparin, histamine, serotonin and DOPA) of mast-cell granules were present and the

* *Heparin* (Richter 111 I.U./mg); *histamine* (Reanal); *serotonin-creatinine sulphate* (Koch-Light); *DOPA* (United Works of Pharmaceutical and Dietetic Products).

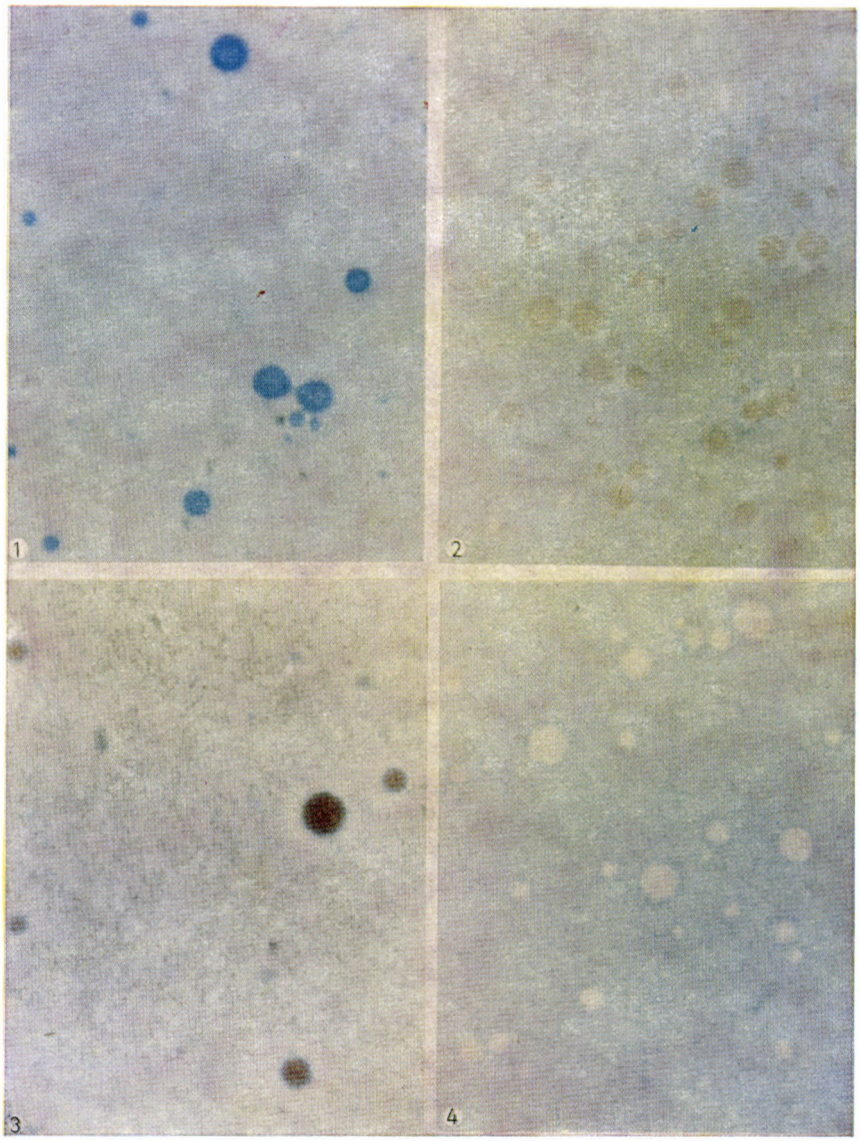

Fig. 1. Model of granules containing heparin, histamine, serotonin and DOPA (1 : 1 : 0.9 : 0.1). The granules are blue. Alcian blue–safranin. × 630

Fig. 2. Model of granules containing heparin, histamine, serotonin and DOPA (1 : 1 : 0.1 : 0.1). The granules are red, the intermediate substance is blue. Alcian blue–safranin. × 630

Fig. 3. Model of granules containing heparin, histamine, serotonin and DOPA (3 : 1 : 0.1 : 0.1). The granules are red, surrounded by an extremely narrow blue zone. There is a very finely granular substance between the granules. Alcian blue–safranin. × 630

Fig. 4. Model of granules containing heparin, histamine, serotonin and DOPA (1 : 1 : 0.1 : 0.1). No staining with alcian blue. × 630

Table 1

The composition of the mixtures applied in the experiments
(The mixtures in which precipitation appeared are enframed)

Heparin	9	7	5	3	1	0
Histamine	1	1	1	1	1	1
Serotonin	0.1	0.1	0.1	0.1	0.1	0.1
DOPA	0.1	0.1	0.1	0.1	0.1	0.1
Heparin	1	1	1	1	1	1
Histamine	9	7	5	3	1	0
Serotonin	0.1	0.1	0.1	0.1	0.1	0.1
DOPA	0.1	0.1	0.1	0.1	0.1	0.1
Heparin	1	1	1	1	1	1
Histamine	1	1	1	1	1	1
Serotonin	0.9	0.7	0.5	0.3	0.1	0
DOPA	0.1	0.1	0.1	0.1	0.1	0.1
Heparin	1	1	1	1	1	1
Histamine	1	1	1	1	1	1
Serotonin	0.1	0.1	0.1	0.1	0.1	0.1
DOPA	0.9	0.7	0.5	0.3	0.1	0

effect of the cell itself was eliminated. If the pure solutions or the four components were mixed, no visible change appeared, whereas macroscopic precipitation could be observed when protein (gelatin) was also present. The precipitation may be considered analogous to the formation of mast-cell granules, i.e., an indicator of linkage between the components. If so, an appropriate histochemical technique is necessary to estimate the maturation stage of the granules formed when the interproportion of the components is varied.

Such a histochemical method is available. It consists in staining with alcian blue-safranin, a method described by SPICER [13, 14], used also by COMBS and co-workers [5] and by the present authors [7]. The procedure was modified and thus stabilized by BALOGH [2]. At the given pH, the two components of the dye stain the granules of mast-cells differently.

The experiments yielded quite surprising results. Similar experiments have surely been delivered by other workers, too [10], though perhaps with the difference that they did not add protein (gelatin), thus failed to obtain macroscopic precipitation. Macroscopic precipitation in given mixtures points to the fact that the formation or non-formation of model granules depends on

the relative concentrations of the components. The model granules invariably appeared whenever the proportions were not highly divergent from those described in literature [10]. The interproportions favourable for granule formation are not too strictly limited: whether the ratio of heparin and histamine was 1 : 1 or 3 : 1, precipitation occurred consistently, and only the morphological pattern seemed to have been influenced. No granules were, however, formed if heparin or histamine was present in great excess. Excessive amounts of serotonin or DOPA did not prevent granules from being formed. Supposedly, the formation of granules is influenced by physico-chemical factors. It is further possible that each component of the granules is separately synthesized in competent cells, and that, as soon as a favourable interproportion has been reached granule formation will start. It may also be supposed that in case of an unfavourable interproportion, e.g., if equal amounts of the constituents are produced in potential mast-cells, granules are not formed at all. We feel, therefore, safe in concluding that there exists a system in which the presence of a primary component — having reached a certain level — induces the synthesis of the other components or their uptake from the surroundings while arresting its own synthesis.

Whenever the amount of heparin exceeded or was equal to that of histamine, the model granules (if formed) gave positive reaction with safranin. The model granules were, on the other hand, invariably alcian blue-positive whenever a relative preponderance of biogenic amines was present, provided their excess was not of a degree to prevent granule formation. We succeeded in producing intermediate forms by changing the quantitative composition stepwise.

Finally, we attempt to draw conclusions to the nature of the granules from the known histochemical reaction. There is no doubt [5, 7] that the granules of young mast-cells stain well with alcian blue. Since in the present model experiments the preponderance of biogenic amines resulted in alcian blue-positive granules, it may be supposed that in young immature mast-cells, too, biogenic amines are predominant. This seems to support our earlier theory [6, 7] that serotonin or histamine are the primary components of mast-cell granules, and that it is the appearance of these substances (or one of them) which induces the synthesis or uptake of heparin.

Characterizing the histochemical reaction by the mixture of substances present in (i.e., added to) the model, in which positive granules are formed, we may state that the alcian-blue-positive component indicates a preponderance of biogenic amines, while the safranin component indicates a preponderance of heparin. It should be noted that, irrespective of the preponderance of biogenic amines, the alcian blue reaction remains negative in the absence of heparin. The safranin reaction is, on the other hand, positive even if no biogenic amines are present.

Of course, the protein that in the mast-cells enables granule formation [11] is not gelatin. Presumably it is a basic protein. Obviously, no model can perfectly reproduce *in vivo* conditions. Considering, however, that the phenomena observed in a model elaborated in living cells [6] were consistent with those observed in the present experiments, it is justified to assume that the phenomena occurring in mast-cells are essentially the same as those observed in the test tubes.

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ENTSTEHUNGSMECHANISMUS DER MASTZELLENKÖRNCHEN

II. Zellfreies Modell

Im Experiment an in-vitro-Modellen der Mastzellenkörnchenbildung wurde festgestellt, daß für das Auftreten der Körnchen ein bestimmtes Verhältnis der einzelnen Komponenten erforderlich ist. Eine bedeutende Verschiebung dieses Verhältnisses macht die Entstehung der Körnchen unmöglich. Die Alcianblau-Komponente der Alcianblau—Safranin-Färbung zeigt das relative Übergewicht biogener Amine an, das für die jungen Körnchen charakteristisch ist. Die reifen Körnchen sind Safranin-positiv. Dies ist ein Hinweis auf das relative Übergewicht des Heparins.

МЕХАНИЗМ ОБРАЗОВАНИЯ ЗЕРНЫШЕК ТУЧНЫХ КЛЕТОК

II. Безклеточная модель

В экспериментах на модели изучалось образование зернышек тучных клеток ***in vitro***. Установлено, что для появления зернышек необходимо определенное соотношение отдельных компонентов. Значительный сдвиг этого соотношения препятствует возникновению зернышек. Компонент альциановый синий при окрашивании альциановым синим + сафранином указывает на относительный перевес биогенных аминов в зернышках. Это характерно для молодых зерен. Зрелые зернышки дают положительную реакцию с сафранином, показывающую относительный перевес гепарина.

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MECHANISM OF THE FORMATION OF MAST-CELL GRANULES

III. SELF-REGULATING SYSTEM OF GRANULE FORMATION

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Synopsis

The effect of actinomycin D and chloramphenicol has been studied in cultures of thymic tissue obtained from normal and cortisone-treated rats. After adding the components of mast-cell granules to the culture medium, their granulopoietic effect was determined by histochemical and fluorescence methods after or without preceding antibiotic treatment. Serotonin and histamine were found to induce heparin synthesis, a process that could be inhibited by antibiotics. Heparin did not induce the biosynthesis of serotonin and histamine. The direction of induction chain is thus serotonin or histamine → heparin and never vice versa.

Introduction

Two models illustrating the formation of mast-cell granules were elaborated in earlier experiments [5, 7], one in tissue cultures, the other in test tubes devoid of cells. Observations made in thymus cultures [5] showed that, unlike serotonin or histamine, heparin alone failed to produce mast-cell granules. In a cell-free model containing four components of mast-cell granules [7] grain-like structures appeared whenever the interproportion of the components remained within certain limits. It was further found that the amount of biogenic amines was preponderant in young granules, while heparin appeared and increased in quantity at a later stage of development.

In the present work actinomycin D and chloramphenicol antibiotics known to interfere with the transcription and translation mechanism, respectively, of the genetic code [4, 10, 11] were employed to determine the synthetic mechanism involved in the formation of mast-cell granules.

Material and method

The thymuses of male Wistar R-rats of 100 g body weight were used. One group of the animals had received cortisone (Adreson — ORGANON) for three days before having been sacrificed, another group served as untreated control.

Tissue cultures were prepared in H-tubes on cockerel plasma coagulated with chick embryo extract; the liquid culture medium contained eight volumes of Parker 199 and two volumes of horse serum. The cultures were washed every second day. Actinomycin D [2] in a final concentration of 0.05 μg/ml or chloramphenicol in one of 100 μg/ml was admixed to the

culture medium on the fifth or sixth day depending on the growth rate of the cultures. This was followed two hours later by the addition of serotonin, heparin and/or histamine in a concentration of 0.1 μg/ml. One group of cultures served as control. Culture media containing chloramphenicol and one of the components of mast-cell granules were exchanged at intervals of 24 hours, those containing actinomycin D were exchanged once, 48 hours after the first treatment. The cultures were fixed in Carnoy's fluid 96 hours after the first treatment and, following the usual microtechnique, stained with toluidine blue (pH 6). The appearance of metachromasia was interpreted as the presence of intracellular heparin.

The medium of the other cultures was soaked up by a filter paper 96 hours after the first treatment, the cultures were then dried on silica gel, fixed for an hour in paraformaldehyde vapour of 80° C and subjected to fluorescence-microscopic analysis.

The number of tissue cultures examined in the present study totalled 520.

Results

While retarding growth, actinomycin D left the major part of cells intact. The employed doses of chloramphenicol had no effect on growth.

Addition of heparin to the culture medium was followed by the appearance of numerous metachromatic cells (Fig. 1). A similarly large number of metachromatic cells was registered 96 hours after the administration of histamine (Fig. 2) or serotonin.

Metachromatic cells arose in cultures whose medium contained heparin in excess whether they had or had not been treated with actinomycin D or chloramphenicol (Fig. 3). No metachromasia was registered in cultures treated with actinomycin D or chloramphenicol if the medium contained only serotonin and histamine (Fig. 4), although the mast-cells of the mother piece were not destroyed by the antibiotics.

Compared to the controls (Fig. 5), the number of cells displaying serotonin fluorescence (Fig. 6). was found to have notably increased in cultures prepared from the thymus of rats pretreated with cortisone. Addition of actinomycin D (Fig. 7) or chloramphenicol to the cultures had no effect in this respect.

Discussion

It is well known that actinomycin D interferes with the transcription from DNA to RNA. Chloramphenicol acts at the level of translation, inhibiting the development of polysomes. In conclusion in both cases enzyme synthesis is prevented. There is experimental evidence [1, 2] that the two antibiotics exert this effect in tissue cultures as well, and, in particular, the inhibitory effect of chloramphenicol on histamine synthesis has been demonstrated [12].

In the synthesis of mast-cell granules a number of enzymes are involved, viz., in the heparin synthesis sulphurase, APS kinase and sulphokinase [13], in the histamine synthesis histidine decarboxylase [3, 9, 13], and in the serotonin synthesis 5-hydroxy-tryptophan decarboxylase [3, 8, 9, 13].

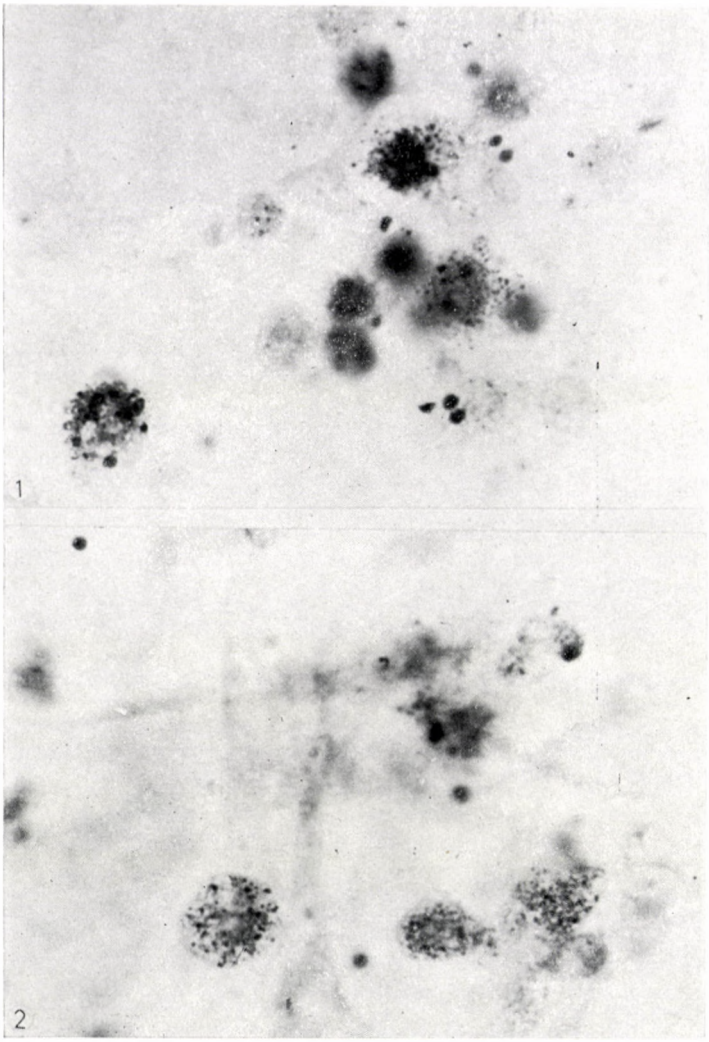

Fig. 1. Cells in thymus culture 96 hours after treatment with heparin. Note the metachromatic cells. Toluidine blue. ×400
Fig. 2. Cells in thymus culture 96 hours after treatment with histamine. Toluidine blue. ×400

When granule components were not included in the medium, the serotonin fluorescence was inhibited neither by actinomycin D nor by chloramphenicol. One might thus believe that serotonin synthesis requires no enzyme. However, this would be inconsistent with well-acceptable literary data [3, 8, 9, 13]. It is more probable that in the presence of the antibiotics, which are known to block enzyme synthesis in general, the synthesis of both 5-hydroxyryptophan decarboxylase and monoamino oxidase, latter being able to de-

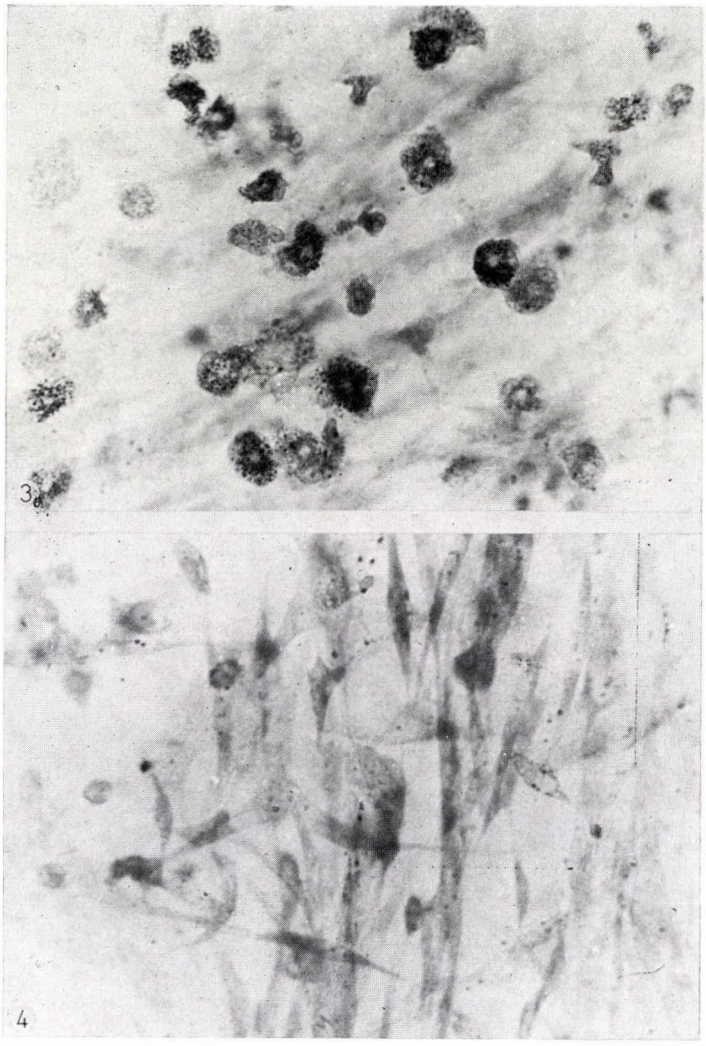

Fig. 3. Cells in thymus culture 96 hours after treatment with chloramphenicol and heparin. Intact cells contain metachromatic granules. Toluidine blue. ×250

Fig. 4. Cells in thymus culture 96 hours after treatment with chloramphenicol and histamine. Though intact, the cells of the zone of migration contain no metachromatic granules. Toluidine blue. ×250

compose serotonin, is blocked. Simultaneous blocking of the antagonistic enzymes may result in a continuously normal serotonin level.

When, on the other hand, serotonin, histamine and heparin were included in the medium, these components were available for the cells without synthesizing them. The fact that the fluorescence of serotonin-treated cultures was found to have increased and, further, that in the heparin-treated cultures a large

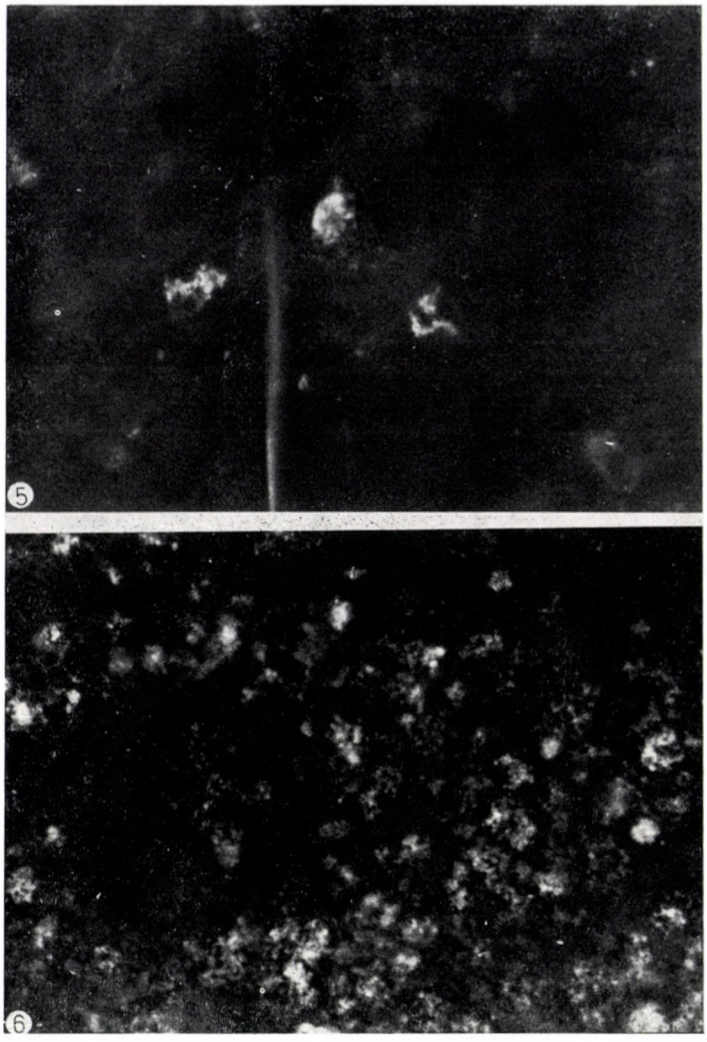

Fig. 5. Cells in the culture of thymic tissue obtained from normal animal. Only few cells show intensive fluorescence. One-hour fixation in paraformaldehyde. × 200
Fig. 6. Fluorescence picture of culture prepared from thymus of rat treated with cortisone over 3 days. Nearly all cells of the migration zone are vividly fluorescent. One-hour fixation in paraformaldehyde. × 160

number of metachromatic granules appeared, justify the conclusion that the employed antibiotics failed to prevent the cells from incorporating these substances.

Adding of serotonin or histamine to antibiotic-free cultures was followed by the appearance of metachromatic granules in the cells, a phenomenon indicating that the uptake of serotonin or histamine induced heparin synthesis

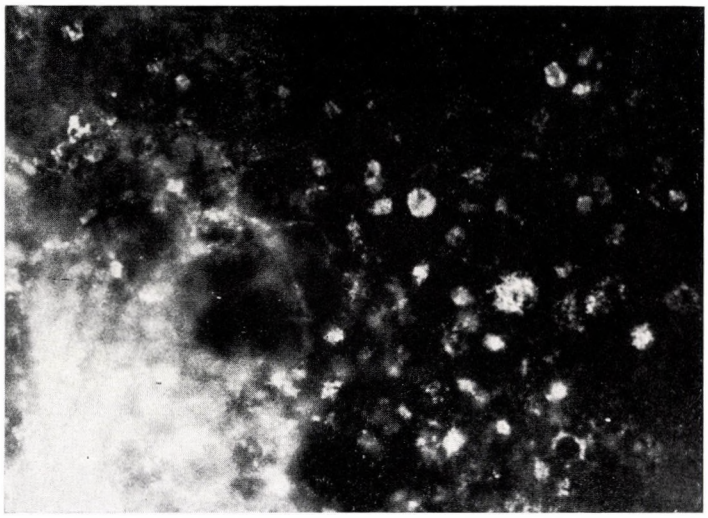

Fig. 7. Fluorescence picture of culture prepared from thymus of cortisone-treated rat, 96 hours after treatment with actinomycin D. Nearly all cells are fluorescent. One-hour fixation in paraformaldehyde. ×160

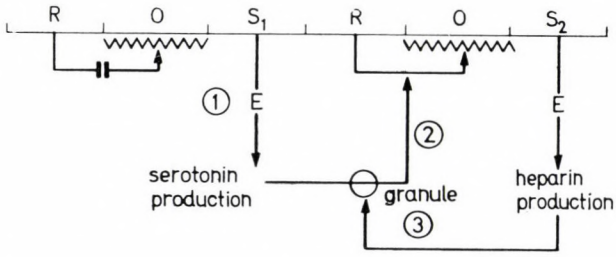

Fig. 8. Self-regulating apparatus of mast-cell-granule formation. The presence of serotonin induces heparin synthesis. Production of serotonin discontinues after it has reached a certain level. R = regulator gene; O = operator gene; S_1 and S_2 = structure genes; E = enzyme. Numbers indicate the order of processes

from the substances contained in the culture medium. No such phenomenon occurred in the presence of antibiotics. It is, thus, safe to conclude that heparin synthesis following the administration of serotonin or histamine requires an enzyme, or enzymes, whose production is inhibited by both antibiotics.

Our earlier experiments [5] made it clear that the presence of heparin does not induce the synthesis of serotonin or histamine in cultured cells; granulopoiesis was studied by electron microscopy, and it was found that empty vacuoles without any trace of serotonin were formed under the effect of heparin. It follows that heparin has not the power to start the synthesis of serotonin or histamine, while these are able to induce the production of heparin.

The experiments present, thus, evidence to show that serotonin or histamine are the primary substances in the formation of mast-cell granules in the rat: they induce the synthesis of heparin, but not *vice versa*. It is known from our previous experiments that no formation of granules takes place unless there exists a certain interproportion between their components. We may therefore assume that heparin synthesis begins and the synthesis of serotonin is arrested as soon as serotonin has reached a certain level.

It is thus presumable (Fig. 8) that a quantitative and qualitative self-regulating system, representing a chain of induction, exists in mast-cells. First serotonin is synthesized and this starts the synthesis of histamine and heparin. The production of granule components is discontinued as soon as a certain interproportion has been established.

The grave problem of what it is that induces the synthesis of serotonin is still to be solved. Both our earlier [6] and present experiments provide evidence to suggest that all cells in which serotonin synthesis has begun are potential mast cells and are sure to develop into true mast cells. It is, however, still to be elucidated how such potential mast cells are brought about, which is the first impulse for the synthesis of serotonin. Investigations in this respect are now in progress.

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ENTSTEHUNGSMECHANISMUS DER MASTZELLENKÖRNCHEN

III. Autoregulationssystem der Körnchenbildung

Die Wirkung von Actinomycin D und von Chloramphenicol wurde an Thymuszellenkulturen normaler bzw. Cortison-behandelter Ratten untersucht. Nach Zugabe der einzelnen Komponenten von Mastzellenkörnchen zur Nährlösung der Kulturen, wurde mit histochemischen Methoden sowie mit Fluoreszenzverfahren die Wirkung der einzelnen Komponenten auf die Körnchenbildung mit oder ohne Antibiotikumbehandlung festgestellt. Serotonin und Histamin induzieren eine Heparinsynthese. Mit Antibiotika läßt sich diese Wirkung abwehren. Heparin bewirkt in den Zellen keine Serotonin- oder Histaminsynthese. Somit geht die Induktionskette der Körnchenbildung jeweils in Richtung: Serotonin oder Histamin → Heparin und niemals umgekehrt.

МЕХАНИЗМ ОБРАЗОВАНИЯ ЗЕРНЫШЕК ТУЧНЫХ КЛЕТОК

III. Авторегуляционная система образования зернышек

Действие актиномицина-Д и хлорамфениколя изучалось на культурах зобной железы нормальных собак и собак, получавших кортизон. После добавления отдельных компонентов зернышек тучных клеток к питательному раствору культур, с помощью гистохимических методов и флюоресцентного способа оценивалось действие отдельных компонентов на образование зернышек без или после дачи антибиотиков. Серотонин или гистамин вызывают синтез гепарина. Дачей антибиотиков можно предотвратить это действие. Гепарин не вызывает синтеза серотонина или гистамина в клетках. Следовательно, при образовании зернышек индукционная цепь всегда имеет направление: серотонин или гистамин гепарин, а никогда не показывает обратное направление.

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HISTOCHEMICAL EVIDENCE OF DIRECT NEURONAL PATHWAYS IN ANODONTA CYGNEA L.

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Synopsis

The interganglionic localization and size distribution of nerve cells belonging to injured axons were examined in *Anodonta cygnea* L. on the basis of RNA accumulation in the pericaryon after cutting peripheral nerves and interganglionic connections. The results are summarized as follows.

(1) There are direct fibres running to the anterior and posterior adductors both from cerebral and visceral ganglia. Cells giving their fibres to the adductors are located not only at the origin of muscular nerves but also in other parts of ganglia.
(2) Motor fibres of the n. pallialis posterior have their cells only in the visceral ganglion.
(3) From the visceral ganglion direct fibres are running through each cerebrovisceral connectives both to the left and right cerebral ganglia, but fibres originating from the cerebral ganglia are running towards the visceral ganglion only on the ipsilateral side. Crossing of the latter takes place in the visceral ganglion. Between the cerebral and visceral ganglia direct axonal connections exist only through the cerebrovisceral connectives.
(4) Connection between cerebral and pedal ganglia is realized through the cerebropedal connectives, all crossings of these fibres take place only in the pedal ganglia.
(5) There is no direct axonal connection (special fibres passing the cerebral ganglion without interruption) between the visceral and pedal ganglia.
(6) According to the size distribution of cells containing RNA with special localization, appearing after axonal injury, the large cells predominate among the regenerating neurones. It is assumed that these are motor cells of the effector system.

Introduction

The central nervous system (CNS) of *Pelecypoda* consists of three distinct pairs of ganglia located far from one another. Nerves originating from different ganglia innervate the neighbouring tissues and organs. However, according to findings with denervation and stimulation of nerves, the ganglion from which the innervating nerve fibres start is not alone involved in the regulation of different organs [2, 11, 12, 18]. The influence of the farther situated ganglion could be realized theoretically in two ways: (a) the effector fibres pass the ganglion located near the organ without interruption or (b) it acts through one or more interlocated neurones. Several authors have supposed [2, 11, 14] fibres originating in the cerebral ganglion and running to the posterior adductor muscle through the visceral ganglion without interruption. Recently the possibility has emerged that certain fibres originating from the visceral ganglion may also innervate the anterior adductor muscle by direct

fibres [15]. Nevertheless, there is no evidence of direct pathways of fibres in different nerves of Pelecypoda.

The perinuclear accumulation of RNA ("perinuclear ring") in regenerating neurons described in *Periplaneta americana* by COHEN and JACKLET [3] is well demonstrable also in the CNS of Anodonta with malachite-green pyronine staining [13]. This pyroninophil substance is finely dispersed, the nucleolus is enlarged and stains lively with pyronine. This method seems to be suitable to localize the pericaryon of fibres present in injured nerves. Furthermore, it may decide the existence of fibres passing the ganglia without interruption. With these experiments the origin of fibres present in the main nerves was examined to obtain detailed information on the direct neural pathways in Pelecypoda.

Material and method

Anodonta cygnea specimens 14—16 cm in length were used.
The following nerves and connectives were damaged by cutting:
(1) nervus adductoris anterioris (n.a.a.) innervating the anterior adductor muscle.
(2) nn. adductores posteriores (nn.a.p.) running to the posterior adductor muscle.
(3) n. pallialis posterior maior (n.p.p.m.).
(4) cerebrovisceral connective (CVc),
(5) cerebropedal connective (CPc),
(6) cerebrocerebral commissure (CCc).

In cases of (1), (3), (4) and (5) either the left or the right nerve was injured. In each series of experiments 3—6 specimens were used.

Nerves were cut after removing a small part of the shell without injuring the ganglia. Those running to the posterior adductor were transected with a fine scalpel through the sypho, partly damaging the muscle tissue itself.

After 3 days all ganglia (right and left cerebral ganglia, visceral ganglion and pedal ganglia) were fixed in Carnoy solution for 30—60 minutes. Embedding took place with strict orientation in celloidin-paraffin, together with the surrounding connective tissue. A series of sections of 7 μ thickness were prepared from the whole ganglion. The plane of sectioning was strictly kept as follows: cerebral ganglia were cut parallel with the sagittal plane, while visceral and pedal ganglia in approximately horizontal planes parallel with the longitudinal axis of the animal (Fig. 1). Staining was carried out with malachite green (Gurr, No. 315) and with pyronine y (GT Gurr, England) according to BAKER and WILLIAMS [1] as described previously [13].

The localization of nerve cells with signs of regeneration was identified by light microscope, the largest diameter was measured and the number of regenerating cells was counted in each ganglion. For the special arrangement of cells within the ganglia the following method was chosen (Fig. 1):

(1) Serial sections of each ganglion were divided into 3 groups. In case of cerebral ganglia zone I, II and III represents the lateral, intermediate and

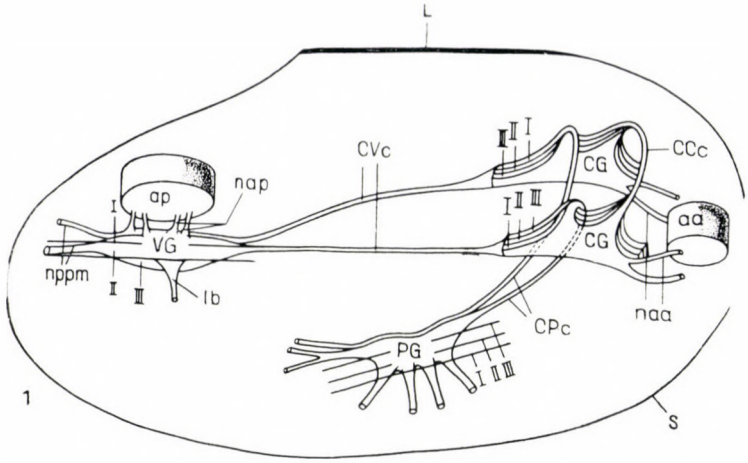

Fig. 1. Scheme of the nervous system of *Anodonta cygnea* unevenly magnified; planes of sectioning are noted, zones of cerebral ganglia are shifted in space. I = zone I, II = zone II, III = zone III; CG = ganglion cerebrale, VG = ganglion viscerale, PG = ganglion pedale, naa = nervus adductoris anterioris, nap = nervi adductores posteriores, nppm = nervus pallialis posterior maior, lb = lobus branchialis, CVc = connectivum cerebro-viscerale, CPc = connectivum cerebro-pedale, CCc = commissura cerebro-cerebralis, aa = musculus adductoris anterioris, ap = musculus adductoris posterioris, S = margin of shell, L = ligamentum

medial third, respectively; in case of the visceral and pedal ganglia the respective zones represent the dorsal, intermediate and ventral thirds.

(2) Regenerating cells were mapped in the two dimensions of the section plane in compliance with the sections belonging to the different zones (Figs 3—8).

Nerve cells were divided according to their size [4] into 3 groups. The percentage distribution of regenerating cells was investigated partly within the different groups and partly related to the size distribution of nerve cells in control specimens. Grouping was based on the limits given in the following Table 1.

Table 1

Grouping of the nerve cells according to their size

	Greatest diameter in μ		
	large	medium-sized	small
Ganglion cerebrale	>26	16—26	<16
Ganglion pedale	>30	20—30	<20
Ganglion viscerale	>34	24—34	<24

Care was taken to avoid repeated counting of cells present in neighbouring sections.

Results

After nerve injury a well-expressed perinuclear ring appears only in large cells [3], where the perinuclear cytoplasm is large enough (Fig. 2a). In smaller cells no such ring was observed but in certain cells other signs of regeneration characteristic of cells with perinuclear ring, were present. These signs are the cytoplasmic pyroninophilia of fine dispersion reminding of that of the ring and the intensive pyronine staining of the enlarged nucleolus, signs never shown by small cells of control animals (Fig. 2b). In our investigations the appearance of this pyroninophilia was observed both in certain smaller and larger cells after nerve injury. The localization of these cells is not accidental; they were present always in the same ganglia and zones consequent to the cutting of nerves in question. For this reason these cells either with or without pyronine ring may be considered as regenerating ones, i.e. cells, whose axons have been damaged. The RNA nature of their pyroninophilia has been proved by ribonuclease digestion [13]. After cutting the nerves recounted in "Methods" the localization of regenerating nerve cells in different part of the CNS was as follows.

When cutting the n.a.a., either left or right, regenerating cells were found both in the cerebral and in the visceral ganglia. Nerve cell with pyronine ring was not present in the pedal ganglia.

When cutting the nn.a.p., regenerating cells were observed in the visceral ganglion and also in both cerebral ganglia.

Cells showing signs of regeneration were present after cutting the n.p.p.m. in the visceral ganglion; after cutting one of the CVc, in the cerebral ganglion of the homolateral side and in the visceral ganglion; after cutting CPc, in the cerebral ganglion of the homolateral side and in the pedal ganglia; after cutting CCc, in both cerebral ganglia and in the visceral ganglion.

In Table 2 data are summarized concerning the size distribution of cells with perinuclear ring and their analogues; furthermore the zonal localization and number of regenerating cells after injuring different nerves are presented. Data of Table 2 are presented also in Figs 3—8, where the actual spatial distribution of cells within the ganglia are demonstrated. Figures are schematic illustrations of zones of ganglia where large, medium and small cells are noted differently.

Equal numbers of regenerating cells were found in both cerebral ganglia after unilateral injury of n.a.a. In the ganglion of the homolateral side these cells were found mainly in zones I and II, near the origin of the n.a.a., but they also occur oppositely (Fig. 3a). The bulk of regenerating cells appeared in the cerebral ganglion of the contralateral side at the origin of CCc in all three zones (Fig. 3b). Cells never form dense groups, but are found rather scattered in the cortical layer of ganglia. In the visceral ganglion such cells were observed

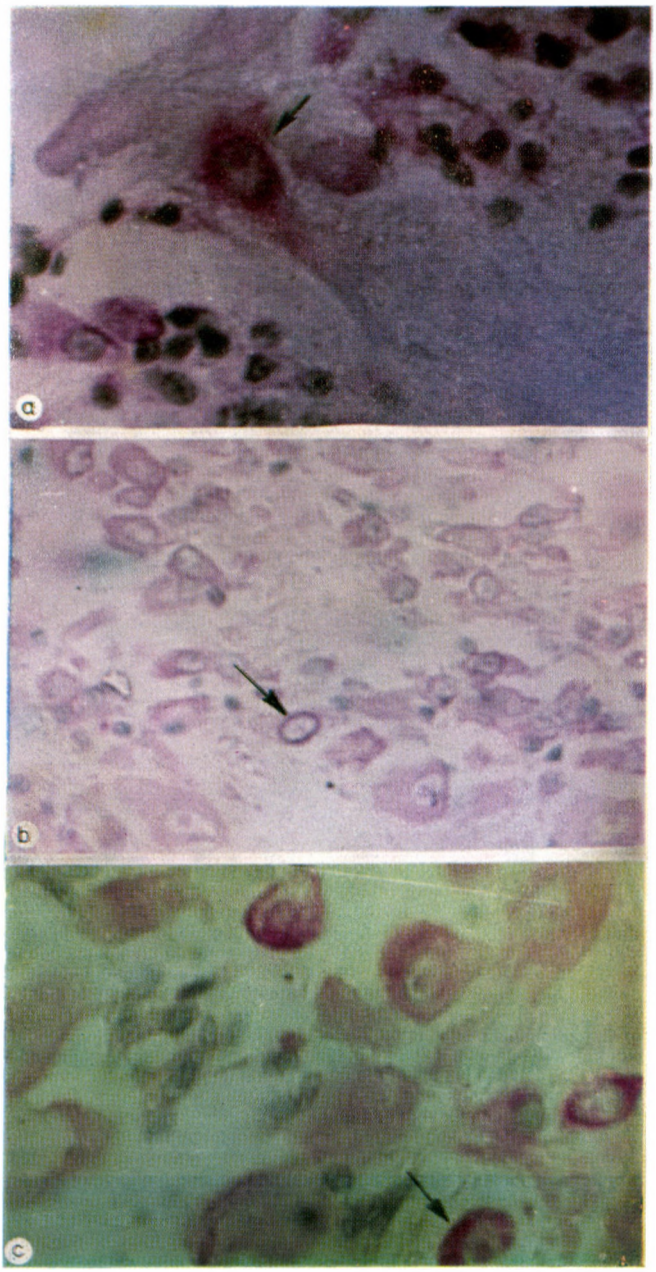

Fig. 2. Regenerating nerve cells; PMaG staining. *a*— large cell (with perinuclear RNA ring), ×750; *b*— small cell with RNA ring, ×750; *c*— small cell without well-expressed ring, ×900

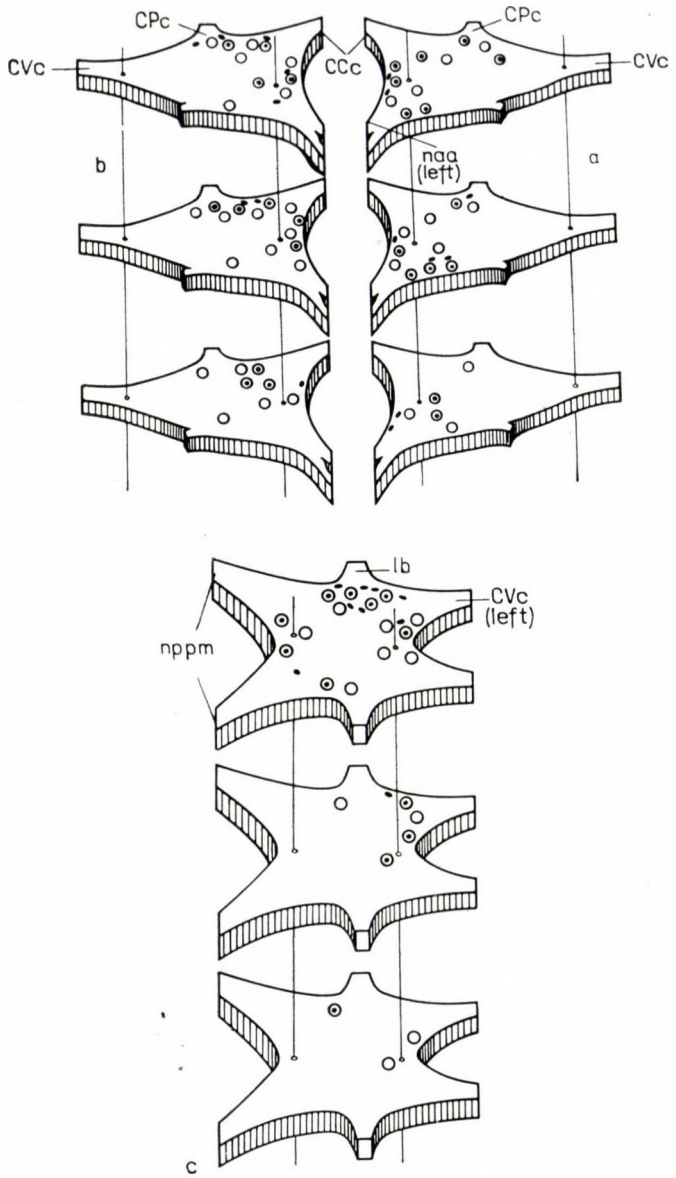

Fig. 3. Regenerating nerve cells after cutting the left n.a. a. *a*— left ganglion cerebrale; *b*— right ganglion cerebrale; *c*— ganglion viscerale; ⊙ = large cell, ○ = medium cell, ● = small cell

mainly in zone I on the ipsilateral side, between the origin of the two CVc and behind the lobus branchialis (Fig. 3c).

After cutting nn. a. p., regenerating cells occupy the place between the two n.p.p.m. and between CVc mainly in zone III (Fig. 4a). Regenerating

Table 2

Distribution of regenerating nerve cells according to size, and zonal localization after cutting different nerves
(Data are averages for 3—6 animals)

Nerve	Ganglion	Zone	Large cell	Medium-sized cell	Small cell
n.a.a. (left)	cerebrale (left)	I	7	4	2
		II	5	5	4
		III	2	3	2
		total	14	12	8
	cerebrale (right)	I	4	6	5
		II	5	8	2
		III	3	5	1
		total	12	19	8
	viscerale	I	8	7	7
		II	3	2	1
		III	1	2	—
		total	12	11	8
	pedale	—	—	—	—
nn.a.p.	cerebrale (left)	I	4	4	3
		II	1	1	1
		III	—	1	—
		total	5	6	4
	cerebrale (right)	I	3	3	2
		II	1	2	1
		III	—	1	—
		total	4	6	3
	viscerale	I	—	3	8
		II	3	6	4
		III	9	8	1
		total	12	17	13
	pedale	—	—	—	—
CVc (left)	cerebrale (left)	I	7	3	2
		II	2	2	1
		III	8	2	—
		total	17	7	3
	viscerale	I	8	13	11
		II	7	20	3
		III	19	15	—
		total	34	48	14
	cerebrale (right)	—	—	—	—
	pedale	—	—	—	—
CCc	cerebrale (left)	I	5	6	3
		II	4	5	2
		III	7	9	—
		total	16	20	5
	cerebrale (right)	I	4	5	1
		II	6	3	3
		III	11	12	3
		total	21	20	7
	viscerale	I	4	3	4
		II	4	1	3
		III	3	3	—
		total	11	7	7
	pedale	—	—	—	—
CPc (left)	cerebrale (left)	I	1	5	1
		II	4	7	4
		III	7	4	3
		total	12	16	8

Nerve	Ganglion	Zone	Large cell	Medium sized cell	Smallicell
	pedale	I	4	10	5
		II	1	6	3
		III	—	3	4
		total	5	19	12
	cerebrale (right)	—	—	—	—
	viscerale	—	—	—	—
n.p.p.m. (left)	viscerale	I	4	7	9
		II	7	4	12
		III	5	14	—
		total	16	25	21
control animals	cerebrale (left)	—	—	—	—
	cerebrale (right)	—	—	—	—
	pedale	—	—	—	—
	cerebrale (left)	—	—	—	—
	cerebrale (right)	—	—	—	—
	viscerale	—	—	—	—
	pedale	—	—	—	—

cells were found also in both cerebral ganglia, near the origin of CVc; the number of these cells is comparatively small, occurring mainly in zone I (Fig. 4b, c). After cutting the left n.p.p.m., regenerating cells are seen in all three zones of the visceral ganglion (Fig. 5). They are localized only in the lateral and medial areas. Similar picture was observed after cutting the right n.p.p.m.

Regenerating cells appear in greatest number when CVc is cut. They are located in the visceral ganglion, mainly at the origin of the injured CVc, but they form groups also in the posterior medial part of the ganglia as well as at the origin of the intact CVc. The majority of these cells was found in zone III (Fig. 6a). In the cerebral ganglion of the ipsilateral side, regenerating cells are seen at the origin of CVc in zone III; in zone I they occur also in different parts of the cortical layer (Fig. 6b). Thus, when cutting the left CVc the pyroninophil cells were found in the visceral ganglion mainly near the origin of the left CVc, while they occur only in the left cerebral ganglion and never in the right one.

After cutting the left CPc regenerating cells were found in greatest number in zones II and III of the ipsilateral cerebral ganglion behind the CPc, but they are also scattered elsewhere (Fig. 7a). Cells of this type appear mainly in zone I in the left pedal ganglion but also in the right one (Fig. 7b).

Cutting of the right CPc was followed by a reversed picture. After damaging the CCc, regenerating cells appeared in both cerebral ganglia mainly at the origin of this commissure and also between this commissure and CPc in zone III (Fig. 8a, b). Regenerating cells are equally found in the visceral ganglion in all three zones, mainly in the posteromedial part (Fig. 8c).

Cells showing regeneration were distributed in all types of injury among the three cell size categories. Considering transections of all kinds in Table 2 the

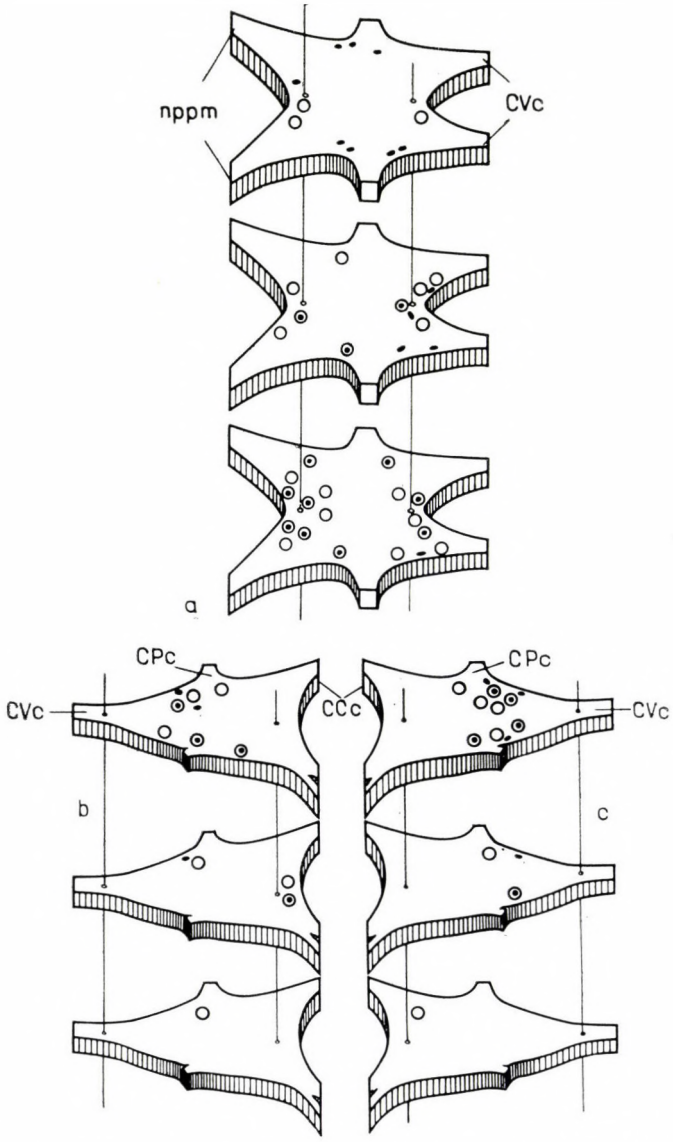

Fig. 4. Cells showing RNA accumulation after transection of nn.a.p. *a*—ganglion viscerale; *b*—right ganglion cerebrale; *c*—left ganglion cerebrale

percentage of regenerating large, medium and small cells per ganglia and per zones is presented. Accordingly 35.6% of large cells were found in the cerebral, 32.6 per cent in the visceral and 11.1% in the pedal ganglia; of the medium cells 45.6% occurred in the cerebral, 41.1% in the visceral and 51.7% in the pedal ganglia; of the small cells 18.8% were found in the cerebral, 26.3% in the visceral and 37.2% in the pedal ganglia. In control specimens the ratio of

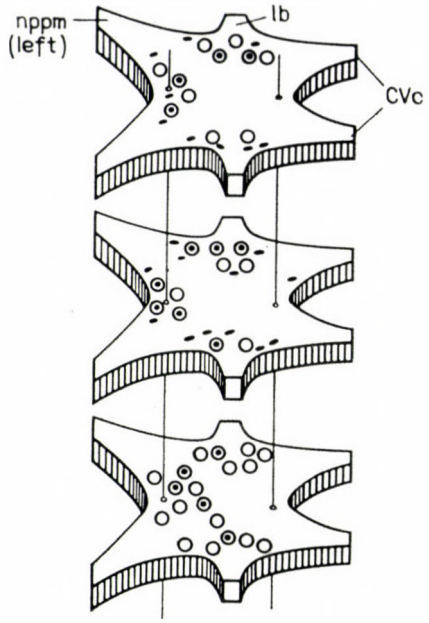

Fig. 5. Regenerating cells in the visceral ganglion after cutting n.p.p.m.

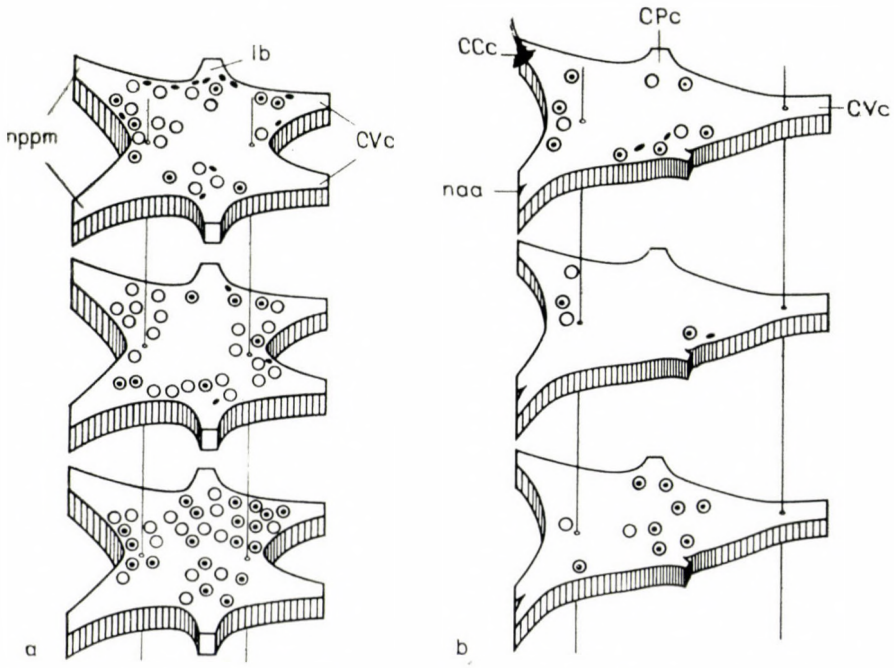

Fig 6. Regenerating nerve cells after cutting the left CVc. *a*—ganglion viscerale; *b*—left ganglion cerebrale

Table 3

Percentage distribution of nerve cells according to size in controls and of regenerating cells in different zones of ganglia after nerve injury

		Ganglion cerebrale			Ganglion viscerale			Ganglion pedale		
	zone	large cells per cent	medium-sized cells per cent	small cells per cent	large cells per cent	medium-sized cells per cent	small cells per cent	large cells per cent	medium sized cells per cent	small cells per cent
Control animals	I	0.9±0.5	29.1± 7.1	70.0± 7.5	0.7±0.6	16.4±7.5	82.9±7.6	3.1±1.1	22.7±4.5	74.2±4.9
	II	2.2±1.7	29.5± 5.6	68.3± 6.7	2.3±3.1	20.0±3.1	77.7±9.8	2.3±0.6	23.3±1.7	74.4±2.3
	III	2.5±1.5	21.6±10.1	63.1±10.2	3.4±1.3	23.5±6.0	73.1±5.5	1.2±0.3	21.2±4.5	77.6±4.6
	mean	1.8	31.1	67.2	2.0	20.0	78.0	2.2	22.4	75.4
Cells with sign of regeneration in animals with nerve injury	I	38.1	40.5	21.3	21.9	30.8	47.3	21.0	53.8	25.2
	II	34.8	40.6	24.6	34.8	37.5	27.7	10.0	60.0	30.0
	III	34.0	55.7	10.3	41.2	54.9	3.9	2.4	41.4	56.2
	mean	35.6	45.6	18.8	32.6	41.1	26.3	11.1	51.7	37.2

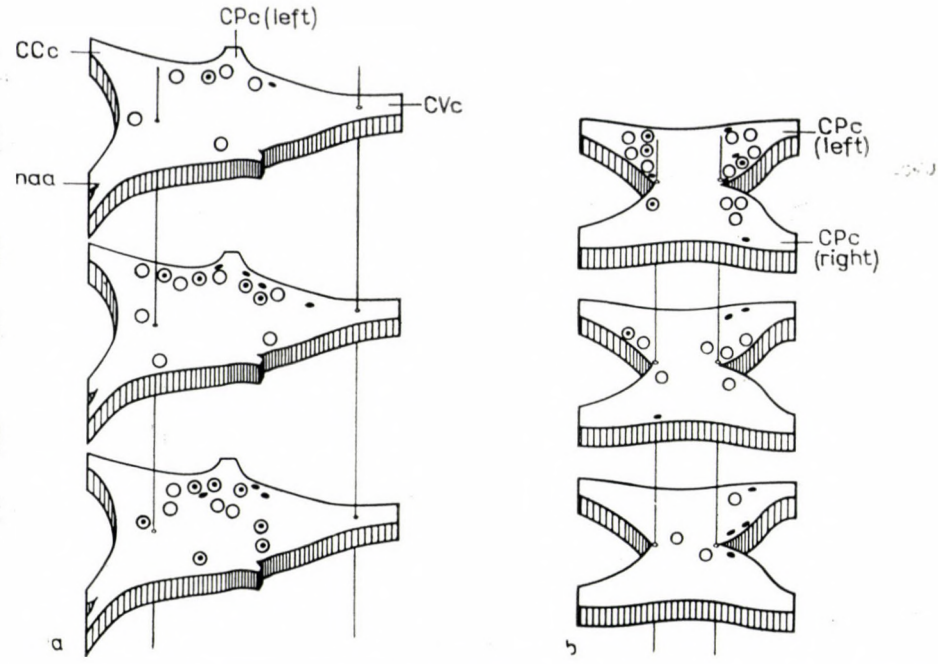

Fig. 7. Distribution of regenerating cells after cutting the left CPc. *a*—left ganglion cerebrale; *b*—left ganglion pedale

large, medium and small cells related to the total number of nerve cells was: 2%, 20—30% and 70—80% respectively (Table 3).

This percentual distribution underlines the fact that when cutting the nerves, the axons of large nerve cells are injured to a higher degree than those of medium cells, while the participation of small cells in the regeneration process is very low as compared to their number.

Discussion

The neuronal network in *Pelecypoda* has hardly been investigated though the knowledge of it is indispensable in the interpretation as well of the centrally regulated coordinated mechanisms as of the peripheral influences on the CNS. HORRIDGE [8] has reported that excitation of the CVc of *Mya* is realized only through transmission in the cerebral ganglia in the anterior adductor muscle and mantle margin, i.e., there are no fibres present passing through the cerebral ganglion. On the contrary, for *Anodonta* direct fibre connections are supposed between CVc and the anterior adductor muscle [15]. No direct axonal connection could be demonstrated between CVc and the posterior pallial nerve [8]

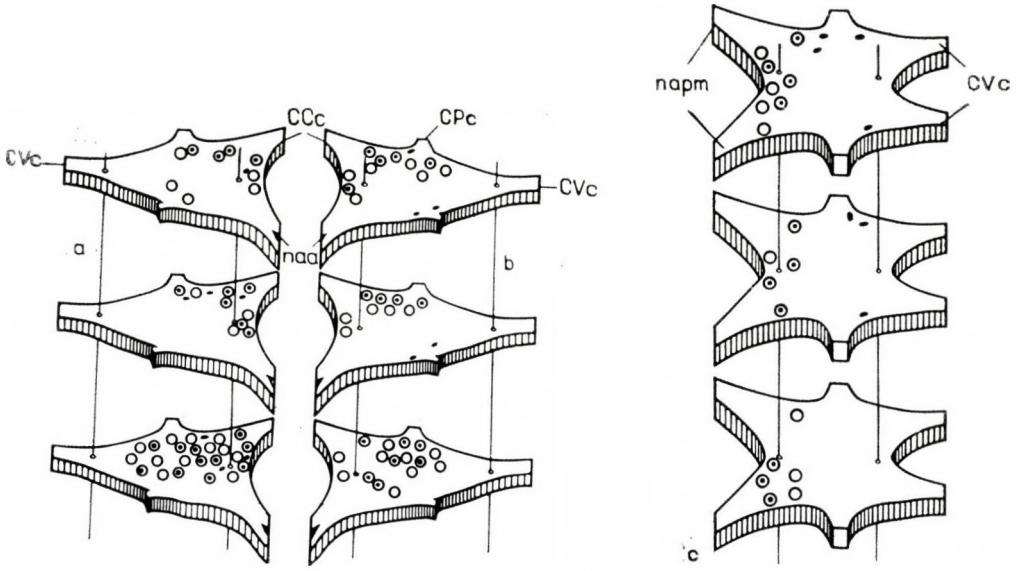

Fig. 8. Localization of regenerating cells after cutting the CCc. *a*—right ganglion cerebrale; *b*—left ganglion cerebrale; *c*—ganglion viscerale

either. In conformity with these findings, MELLON [10] demonstrated in the ganglion viscerale of *Spisula* the origin of efferent fibres of the posterior pallial nerve and of the sypho nerve. He also found in this ganglion the synapses of the afferent fibres and supposed here the presence of a pallial integrative centre.

According to our results, in the CNS of *Anodonta* there are many cells sending their axons to a far-located organ through another ganglion without interruption. These pathways are presented in Fig. 9 in a scheme based on the details of Figs 3—8.

It has been proved that in the innervation of the anterior adductor muscle, besides the fibres of cerebral ganglia, fibres originating from the visceral, and passing through the cerebral ganglia without interruption are involved, contradicting the findings of HORRIDGE [8] in *Mya*. Moreover, axons present in the two nervus adductoris anterioris originate not only in the ipsilateral cerebral ganglion but some of them come from the contralateral one (Fig. 3).

In analogy to this, from both cerebral ganglia direct fibres passing through the visceral ganglion run to the posterior adductor, whereas the posterior adductor muscle is primarily innervated from the visceral ganglion. Direct fibres starting from the cerebral ganglia and running to the posterior adductor occur exclusively in the CVc ipsilateral to the cerebral ganglion in question. However, the crossing of the fibres originating from the two cerebral ganglia may take place in the visceral ganglion (Fig. 4).

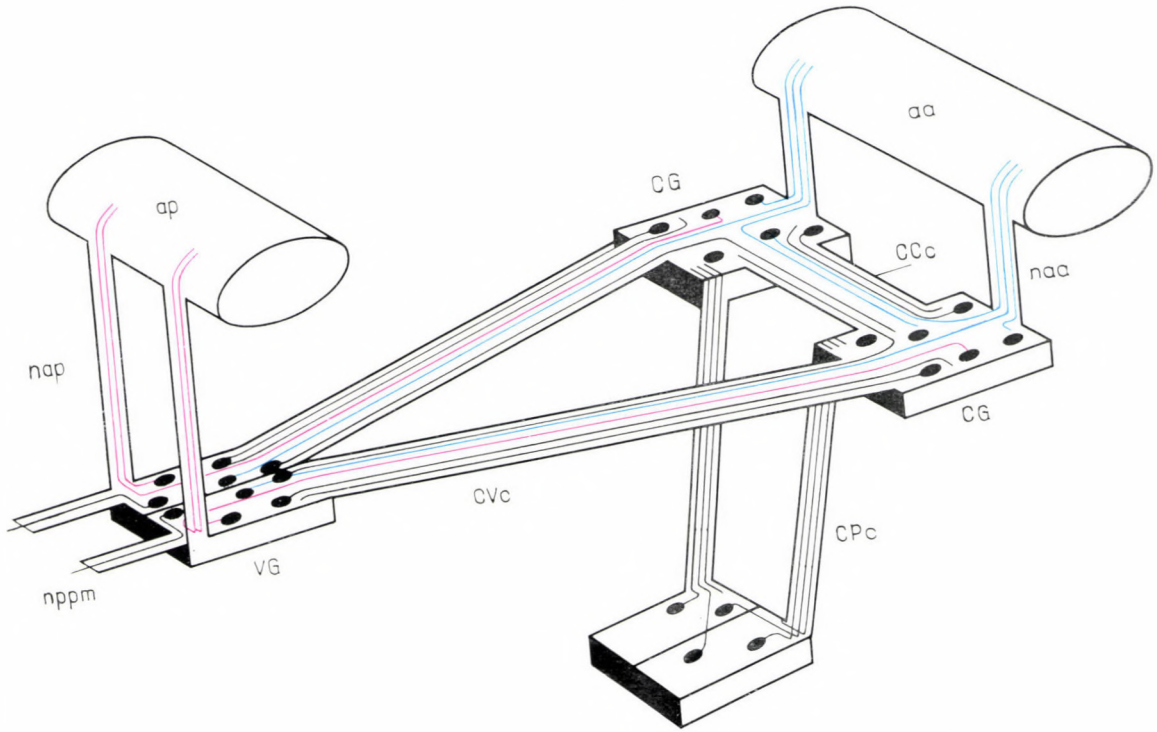

Fig. 9. Direct neuron pathways in *Anodonta cygnea*. Abbreviations, see Fig. 1. Blue = direct fibres running to the anterior adductor muscle; red = direct fibres running to the posterior adductor muscle

The origin of nerves innervating the two adductors assures the morphological basis for both cerebral and visceral ganglia to influence individually and directly each adductor. This way a process taking place in either of the ganglia may influence both adductors simultaneously, without interneural synaptic transmission. These data are supported by conclusions with related physiological investigations [2, 11, 14, 15, 16].

It is also obvious that cutting of one of the nervus adductoris anterioris is not sufficient to eliminate the effect of the cerebral ganglion of the corresponding side if the CCc and the other n.a.a. remain intact (Fig. 9), as it was also supposed in physiological studies [15].

Nevertheless, a part of peripheral nerves has no direct connection with more than one ganglion. This has been demonstrated with n.p.p.m., where an injury results in regenerating cells only in the visceral ganglion. These findings are in good agreement with electrophysiological results of HORRIDGE [8] and MELLON [10]. The crossings of fibres arriving from the two n.p.p.m. are numerous within the visceral ganglion (Fig. 5). This means that (a) the response of the anterior adductor after stimulation of the sypho may be realized only through transmission in the visceral ganglion and (b) stimulation of either side can be transferred by both CVc towards the cerebral ganglia (Fig. 9).

In *Mytilus* WOORTMANN [17] has described the fusion of fibres of the anterior and posterior pallial nerves, whereas along the mantle margin no conduction of impulse could be demonstrated. Our experiments have not proved the presence of fibres in the n.p.p.m. originating from the cerebral ganglion i.e., no direct axonal connection was found between cerebral and visceral ganglia through the pallial nerves.

By investigating the interconnections of cerebral, visceral and pedal ganglia it was found that cerebral ganglia occupy a central position and that the two other ganglia have no direct axonal contacts with each other. Thus, it is obvious that there not only fails to exist any viscero-pedal nerve [7] but also no fibres are passing through the cerebral ganglia which could establish connection without synapses between the visceral and cerebral ganglia (Fig. 9). The connection between cerebral and visceral ganglia is realized through the CVc. It has been shown that only those fibres running in CVc cross between the two cerebral ganglia (through CCc) which originate from the visceral ganglion; those coming from one of the cerebral ganglia may cross only within the visceral ganglion (Figs 6, 8, 9).

Pedal ganglia are connected only with the cerebral ganglia through CPc. No fibres originating from pedal ganglia are found in CCc, in other words, crossings between contralateral cerebral and pedal ganglia take place already between the pedal ganglia (Figs 7, 9).

Our results show that not all the theoretically possible pathways could be demonstrated in the CNS of *Anodonta* in the form of direct fibres.

The fact that the centralization of the nervous system in *Anodonta* is rather low is demonstrated by the relatively high number of direct pathways, by the dispersion of nerve cells belonging to a given pathway within the ganglion and by the multiple axon crossings between the left and right ganglia; this latter had already been observed in impregnated preparations [5].

Regenerating cells are different in size, large and small cells occur always mixed. No reliable data are available whether the differences in size correspond to differences in function. However, the fact that in large cells regeneration occurs in larger proportion as related to the normal distribution (Table 3) shows that these cells are of peripheral function, presumably motor cells.

After cutting the nerves, fewer cells developed definite pyronine rings or other signs of regeneration than the number of the cut fibres. This might be attributed to the large number of internuntial cells or to the extraordinary branching of the axons [5] making the injury of all or nearly all peripheral parts of cells questionable. This latter is presumably important for the appearance of intensive regeneration detectable by the applied histochemical method. It should be mentioned that the manyfold branching of axons results not only in an extremely rich synaptic network but, besides the neurone pathways demonstrated in this work, it may cause variations which cannot be analyzed satisfactorily with the applied method.

Of course, in *Anodonta* only a small proportion of the neurone network consists of mononeuronal pathways and the majority of the pathways are mono- or polysynaptic [8, 10]. The many-sided direct connection of cerebral and visceral ganglia refers to their relative independence. At the same time it is not excluded that these long-running axons have in one or another form a coordinating role, e.g., in the regulation of the synchronous function of adductors [9].

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HISTOCHEMISCHER NACHWEIS DIREKTER NEURONALER BAHNEN BEI *ANODONTA CYGNEA* L.

Die zwischenganglionäre Lokalisation und die Größenverteilung der Nervenzellen lädierter Axone wurden bei *Anodonta cygnea* L. auf Grund der RNS-Akkumulation im Pericaryon nach Durchschneiden der peripheren Nerven und der zwischenganglionären Verbindungen untersucht. (1). Sowohl aus den Cerebral- als auch aus den Visceralganglien gehen direkte Fasern zu den vorderen und hinteren Mm. adductores. Die ihre Fasern zu den Mm. adductores aussendenden Zellen finden sich nicht nur an der Ursprungsstelle der Muskelnerven, sondern auch in anderen Teilen der Ganglien. (2) Die Zellen der motorischen Fasern des N. pallialis posterior finden sich ausschließlich im Visceralganglion. (3) Vom Visceralganglion laufen direkte Fasern über sämtliche cerebro-viscerale Verbindungswege sowohl zum linken als auch zum rechten Cerebralganglion, während die aus dem Cerebralganglion abgehenden Fasern zum Visceralganglion nur auf der ipsilateralen Seite verlaufen. Ihre Kreuzung findet im Visceralganglion statt. Zwischen dem Cerebral- und Visceralganglion bestehen direkte Axonverbindungen nur über cerebro-viscerale Bahnen. (4) Zwischen Cerebral- und Pedalganglien wird die Verbindung über cerebro-pedale Bahnen verwirklicht. Sämtliche Kreuzungen dieser Fasern finden ausschließlich in den Pedalganglien statt. (5) Zwischen den Visceral- und Pedalganglien bestehen keine direkten Axonverbindungen (es gibt keine speziellen Fasern, die das Cerebralganglion ohne Unterbrechung passieren). (6) Auf Grund der Größenverteilung der RNS-enthaltenden Zellen spezieller Lokalisation, die nach Axonschädigung auftreten, ist der Anteil der größeren Zellen in den regenerierenden Neuronen vorherrschend. Es wird angenommen, daß sie motorische Zellen des Effektorensystems darstellen.

ОПРЕДЕЛЕНИЕ НЕПОСРЕДСТВЕННЫХ НЕВРОННЫХ ПУТЕЙ БЕЗЗУБКИ (*ANODONTA CYGNEA L.*) НА ОСНОВАНИИ ГИСТОХИМИЧЕСКИХ ИССЛЕДОВАНИЙ

После перерезки некоторых главных нервных разветвлений и коннективов, связывающих ганглии у беззубки, принимая во внимание накопление цитоплазматической РНК, авторы исследовали внутриганглионарную локализацию клеток поврежденных аксонов, а также распределение клеток по величине. Они получили следующие результаты: (1) Как к передней так и к задней запирательной мышцах идут прямые волокна от

двух церебральных и висцеральных ганглиев (ЦГ и ВГ). Клетки, иннервирующие мышцы, внутри ганглия расположены не только вблизи выхода нерва по направлению к аддукторам, но также находятся и в других частях ганглия. (2) Волокна, идущие в заднем паллиальном нерве, непосредственно связаны лишь с ВГ. (3) Оба церебровисцеральных коннектива (ЦВК), выходящих из ВГ, содержат непосредственные нервные волокна для обоих ЦГ. Однако, волокна, исходящие из ЦГ, направляются к ВГ только лишь через ЦВК соответствующей стороны. Перекрещение последних происходит в ВГ. Непосредственная аксональная связь между ЦГ и ВГ осуществляется только через ЦВК. (4) Связь между церебральными и педальными ганглиями осуществляется посредством двух церебропедальных коннектив. Перекрещение волокон происходит лишь в педальном ганглии. (5) Непосредственной аксонной связи (отдельного нерва или волокон проходящих через ЦГ) между висцеральным и педальными ганглиями нет. (6) Распределение исследованных нервных клеток по величине указывает на то, что регенерация после перерезки нервов в наивысшей степени наблюдается в клетках наибольшего размера. Авторы предполагают, что они являются моторными клетками эффекторной системы.

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AN IRON-ALUM METHOD FOR ELECTIVE DEMONSTRATION OF ACID MUCOPOLYSACCHARIDES I.

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Synopsis

A new method for the elective demonstration of acid mucopolysaccharides is described. Pretreatment with iron alum makes it possible to stain acid mucopolysaccharide-containing structures very electively with otherwise non-elective basic dyes. It is claimed that, owing to their blocking effect, the ions $(FeOH)^{++}$ and $[Fe(OH)_2]^{+}$ are responsible for elective staining.

Introduction

There are numerous methods for the histochemical demonstration of acid mucopolysaccharides.

(1) Certain dyes (e.g. thionine, toluidine blue) visualize acid mucopolysaccharides by metachromasia [5].

(2) Owing to the intensive acidity of acid mucopolysaccharides, otherwise non-elective basic dyes can be made elective at adequately low pH-values [2, 7].

(3) The affinity of some metallic ions (e.g. Ba^{++}) to acid mucopolysaccharides can be utilized by adding a suitable reagent to the mucopolysaccharide—metallic ion complex [12].

(4) Hale's method uses Prussian blue to demonstrate colloidal iron bound by acid mucopolysaccharides [6].

(5) The principle of competitive ion antagonism is utilized in the course of which the bond formation between basic dyes and acid mucopolysaccharides is influenced by the addition of metallic ions to the dissolved dye [10].

(6) The employment of quaternary ammonium bases (e.g. cetylpyridinium chloride) is based on the same principle [9, 14].

(7) The sulphate groups of acid mucopolysaccharides form tetrazonium sulphonates with tetrazonium salts; combined with α-naphthol, these are water-insoluble azo dyes [11].

(8) Combinations of these methods are widely used.

Studying the effect of different metallic ions, we have found that using iron alum as mordant results in elective staining of acid mucopolysaccharides if followed by treatment with a suitable basic dye.

Material and method

Variously fixed (Carnoy, neutral formol, 70% ethanol), paraffin-embedded and also cryostat sections were prepared.

Staining of paraffin sections

(1) The sections were brought to distilled water,
(2) then pretreated with a 5% solution of iron alum at room temperature for 15 minutes,
(3) rinsed with distilled water for 5 minutes,
(4) stained with a basic dye (v. infra),
(5) dehydrated, clarified and mounted as usual.

Staining of cryostat sections

In the case of cryostat sections the procedure started with iron alum pretreatment without foregoing fixation.

Dyes tested for staining

Suitably elective staining was achieved with the following basic dyes.

I. Quinone-imine dyes
  (1) Thiazine derivatives: thionine, azure A, methylene blue, toluidine blue.
  (2) Oxazine derivatives: brillant cresyl blue, celestin blue, Nile blue sulphate, new blue R,
  (3) Azine derivative: safranine T.

II. Copper phthalocyanine derivative: alcian blue 8GS.

III. Phenylmethane dyes
  (1) Triphenylmethane derivatives: basic fuchsin, methyl viole t, crystal violet.
  (2) Diphenylmethane derivative: Victoria blue B.

IV. Nitro- and azo dyes
  (1) Mono-azo dyes: Janus green B, chrysoidin.
  (2) Diazo- and polyazo dyes: Bismarck brown.

V. Xanthene dyes

Acridine dyes: acridine yellow, acridine orange, acriflavine.

One per cent aqueous solutions of these dyes were used. The time of staining varied from 5 to 15 min.

Sections were prepared from the costal cartilages and the larynx of white rats. In the laryngeal sections, besides the cartilage, the mast cells and the mucous glands were examined for staining properties.

For comparative and control purposes in some experiments iron alum was replaced by ferric sulphate, ferric chloride or ferrous sulphate.

Results

Iron-alum pretreatment and demonstration of bound iron

Of the various concentrations of iron alum, those between 1 and 5% proved to be the most suitable. Treatment with iron alum results in $(FeOH)^{++}$ and $[Fe(OH)_2]^{+}$ ions bound by the anions of the tissues. The linkage is extremely strong, so that washing for several hours with running water fails to remove the iron from the surface of the tissues.

When employing mordant-fixed dyes, bound iron is indicated by lake formation [1]. Since this technique is not sufficiently specific, we demonstrated

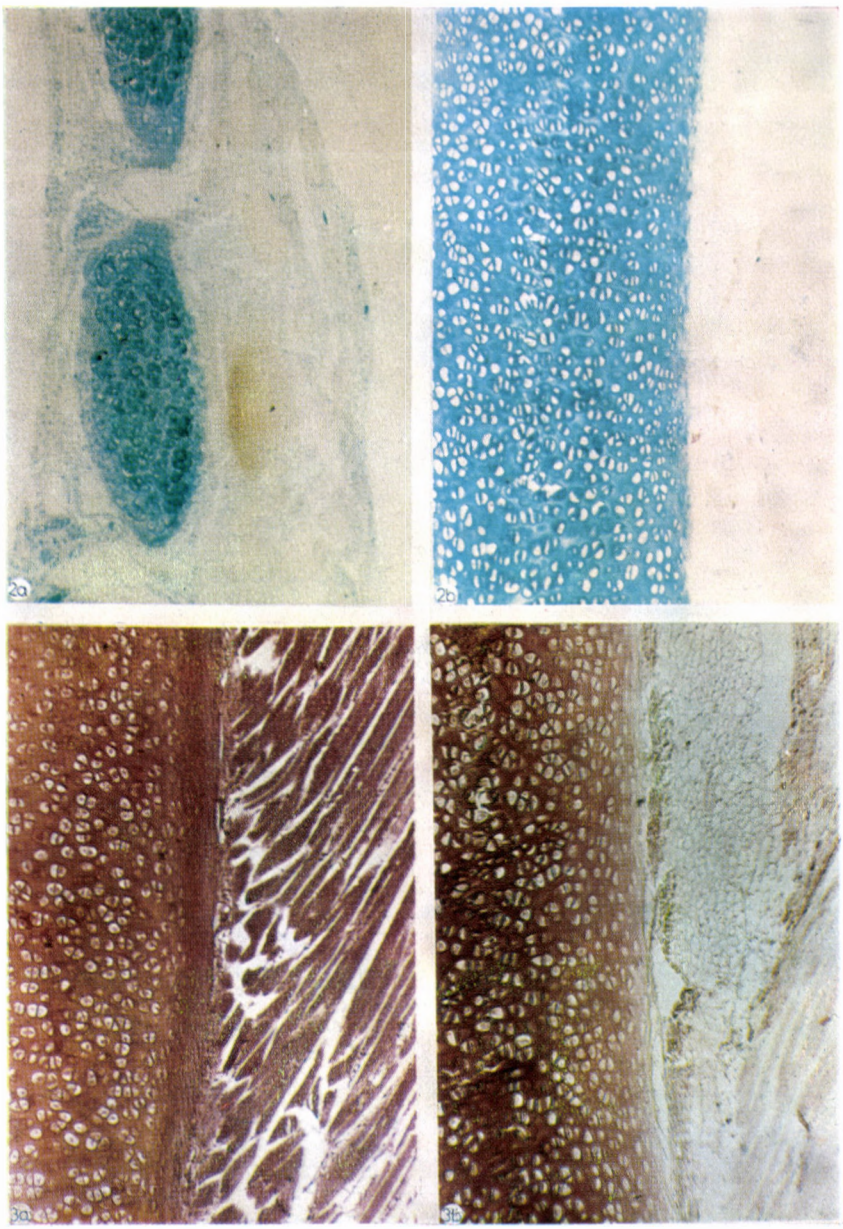

Fig. 2a. Rat larynx. Alcian blue. Control. ×100
Fig. 2b. Rat rib. Iron-alum—alcian blue. ×100
Fig. 3a. Rat rib. Safranin. Control. ×100
Fig. 3b. Rat rib. Iron-alum—safranin. ×100

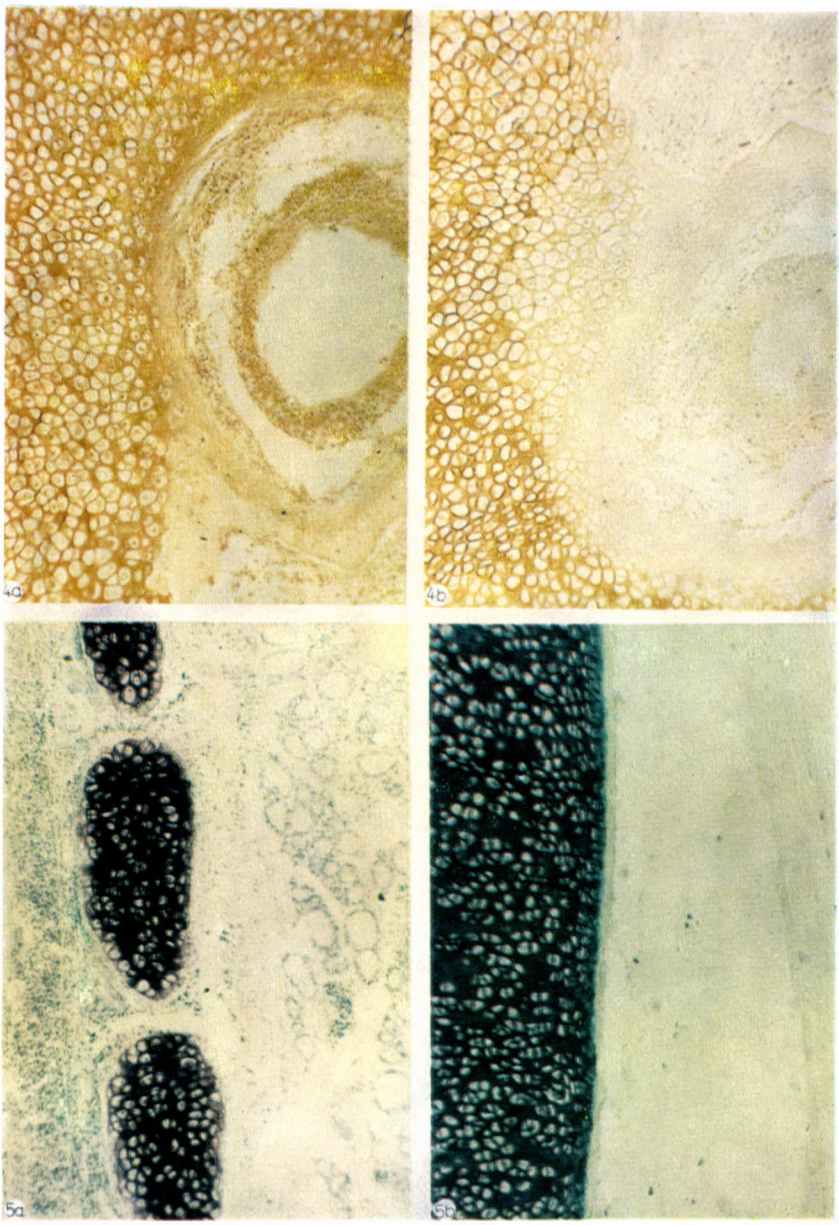

Fig. 4a. Rat larynx. Acridine yellow. Control. ×100
Fig. 4b. Rat larynx. Iron-alum—acridine yellow. ×100
Fig. 5a. Rat larynx. Azure A. Control. ×100
Fig. 5b. Rat rib. Iron-alum—azure A. ×100

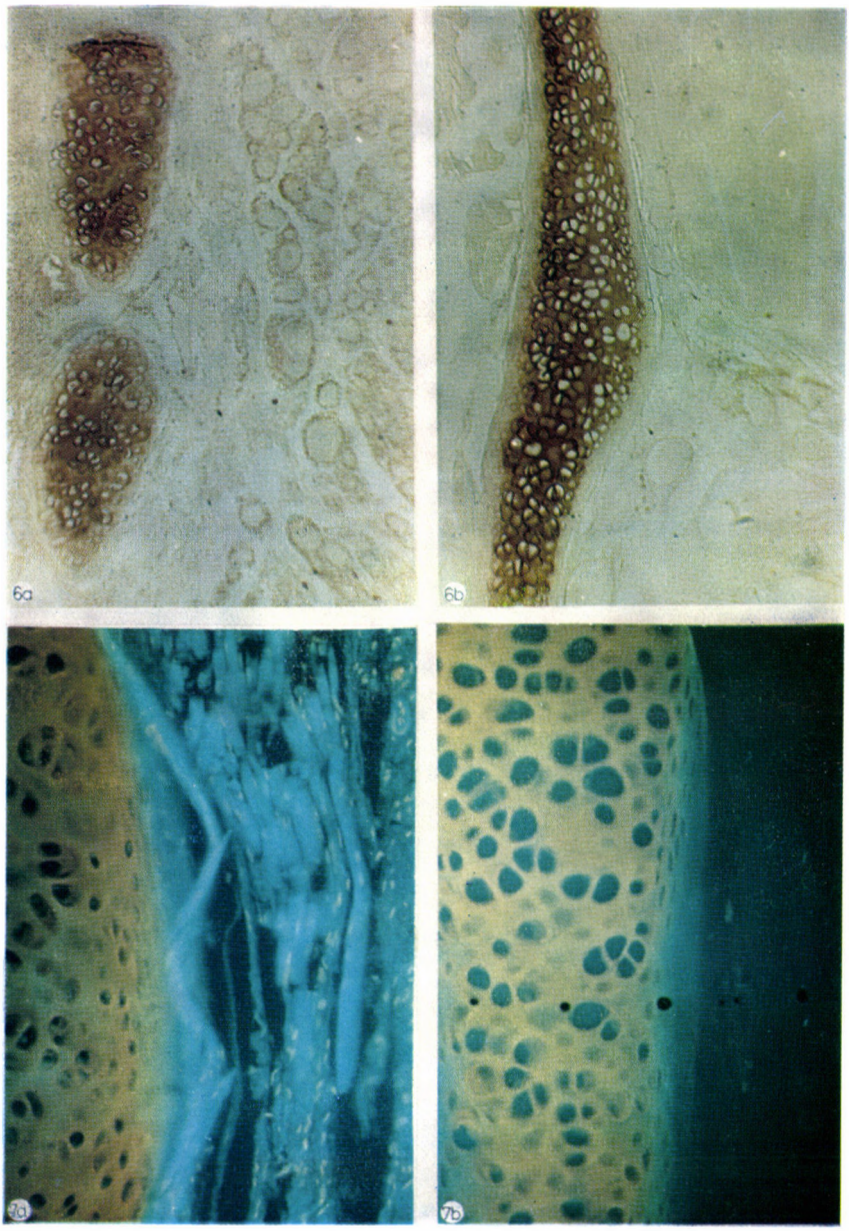

Fig. 6a. Rat larynx. Bismarck brown. Control. ×100
Fig. 6b. Rat larynx. Iron-alum—Bismarck brown. ×100
Fig. 7a. Rat rib. Fluorescence-microscopic picture. Acridine yellow. Control. ×250
Fig. 7b. Rat rib. Fluorescence-microscopic picture.
Iron-alum—acridine yellow. ×250

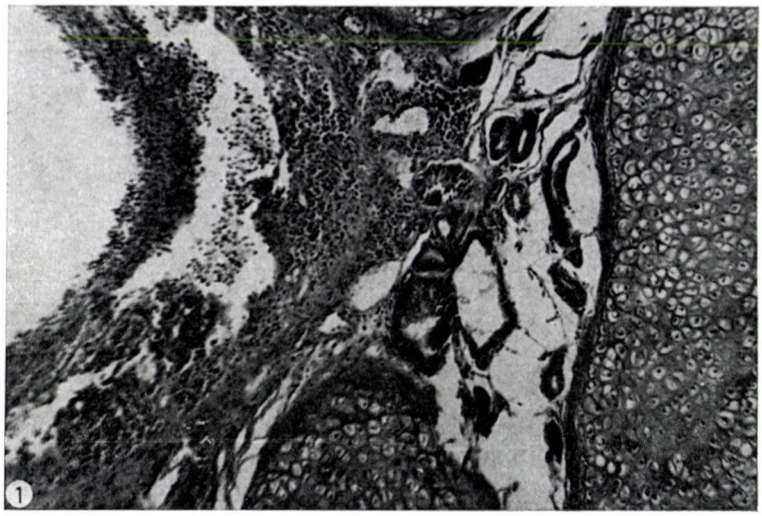

Fig. 1. Rat larynx. Iron-alum ammonium sulphide reaction. 10×10

the presence of bound iron in the form of sulphide or Prussian blue by treating the sections with ammonium sulphide or potassium ferrocyanide [13]. Iron was found to be taken up by all examined tissues. The uptake of iron was most uniform with both methods in the nuclei and in the structures containing acid mucopolysaccharides (Fig. 1).

Staining with various basic dyes

Basic dyes, as enumerated in the foregoing, are known to stain acid mucopolysaccharides orthochromatically or metachromatically but never electively. When staining was preceded by iron-alum treatment, all the structures containing acid mucopolysaccharide were intensively stained, whereas other structures showed no — or hardly any — staining reaction, a phenomenon indicative of electivity (Figs 2 to 6).

Optimum electivity was obtained with azure A, alcian blue, Bismarck brown, acridine yellow, acridine orange, Nile blue, celestin blue, brillant cresyl blue, new blue R, toluidine blue, methylene blue, thionine and Janus green B.

Moderately elective proved to be crystal violet, methyl violet, acriflavine and chrysoidin.

Weak electivity was shown by safranine and Victoria blue.

Considering the fluorochromatic nature of acridine yellow and acridine orange, we examined the effect of iron-alum pretreatment on fluorescence. Fluorescence, too, turned elective (Fig. 7).

Employment of other iron salts

Ferric salts were found to have the same influence on the uptake of basic dyes as iron alum, while pretreatment with ferrous salts failed to yield elective staining of acid mucopolysaccharides.

Discussion

Iron alum is widely used in microtechniques as mordant [1]. The exact mechanism of the mordanting procedures is still obscure. According to recent theories [4], iron from the strongly acid solution of iron alum is bound by the tissues in the form of $(FeOH)^{++}$ and $[Fe(OH)_2]^{+}$ ions. WIGGLESWORTH [13] suggests that, of the tissue anions, phosphate and carboxyl groups are mainly responsible for the uptake of iron hydroxide ions. Other investigations [12] have shown that the sulphate groups of acid mucopolysaccharides, too, are capable of binding metal ions. Our experiments have confirmed this phenomenon in respect of iron hydroxide ions.

When staining is done by mordant-fixed dyes the iron hydroxide ions, combining with a certain group of the dye, produce a lake. The chelate-forming groups of the dye molecules have proved to be chiefly responsible for lake formation [1]. Mordant-fixed dyes do not, of course, yield elective staining because of the diffuse character of the iron hydroxide uptake and the lake formation. It is worthy of note that lake-forming dyes (e.g. hematein, carminic acid, oxazine) are amphoteric.

It follows from the foregoing that the main difference between the use of iron alum as mordant in general and our method in particular is the electivity of our method. The detailed mechanism of this electivity requires further investigation. Our observations in this respect encourage us nevertheless to propose the following ideas.

(1) The iron-hydroxide ions which are undoubtedly responsible for the elective staining of acid mucopolysaccharides are taken up also by other tissues. We suppose that — with the exception of acid mucopolysaccharide-containing structures — the tissue anions are permanently blocked by the positively charged iron-hydroxide ions so that they become inaccessible for basic dyes. There is, in this respect, competitive antagonism between the iron-hydroxide ions and the basic dyes. This view is supported by the fact that, if staining is followed by treatment with iron alum, the dye is displaced from the acid mucopolysaccharide-free tissues.

(2) Since, as has been noted, chemically bound ions of iron hydroxide are to be found in acid mucopolysaccharides also, it is rather difficult to tell how they take up basic dyes after iron-alum mordanting. One can only speculate in this respect.

(a) It seems plausible to suppose that elective staining is due to differences in the strength of iron binding. While the molecules of basic dyes are capable of displacing weakly bound iron hydroxide ions from the anions of the acid mucopolysaccharides, they are unable to expel such ions if they are bound, e.g. by phosphate ions.

(b) It is, on the other hand, possible that the iron-hydroxide ions do not block all dye-binding anions in the acid mucopolysaccharides. The problem of where and how strongly the iron-hydroxide ions are bound by the acid mucopolysaccharide complex is to be elucidated.

Investigations to shed light on the mechanism of the electivity of the staining described in the present paper are in progress.

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METHODE ZUM ELEKTIVEN NACHWEIS SAURER MUCOPOLYSACCHARIDE MIT EISENALAUN. I.

Eine neue Methode zum elektiven Nachweis saurer Mucopolysaccharide wird mitgeteilt. Das Wesen des Verfahrens besteht darin, daß, nach Eisenalaun-Vorbehandlung der Schnitte, die verschiedenen — ansonsten keine elektive Färbung bewirkenden — basischen Farbstoffe die saure Mucopolysaccharide enthaltenden Strukturen optimal elektiv zum Vorschein bringen. Auf Grund der Ergebnisse sind für die elektive Färbung die $(FeOH)^{++}$- und die $[Fe(OH)_2]^{+}$-Ionen durch ihre blockierende Wirkung verantwortlich.

МЕТОД ДЛЯ ИЗБИРАТЕЛЬНОГО ВЫЯВЛЕНИЯ КИСЛЫХ МУКОПОЛИСАХАРИДОВ ЖЕЛЕЗНЫМИ КВАСЦАМИ. I.

Дается описание нового метода для избирательного выявления кислых мукополисахаридов. Сущность метода состоит в том, что после предварительной обработки срезов железными квасцами, различные — впрочем не дающие избирательного окрашивания — основные красители выявляют структуры, содержащие кислые мукополисахариды, с максимальной избирательностью. Согласно полученным результатам, соответственными за избирательное окрашивание являются ионы $(FeOH)^{++}$ и $[Fe(OH)_2]^{+}$ посредством своего блокирующего действия.

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DEMONSTRATION OF ACID MUCOPOLYSACCHARIDES BY BENZIDINE REACTIONS

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Synopsis

Two new methods for the histochemical demonstration of acid mucopolysaccharides are presented. The molybdate-reduction method is based on the fact that, in the course of treatment with phosphomolybdic acid, molybdate ions bound by the protein component of acid mucopolysaccharides are transformed into molybdenum-blue under the reducing effect of benzidine. The benzidine-oxidation method is based on that in the presence of hydrogen peroxide, ferric and certain other metallic ions bound by the tissue structures catalyse the oxidation of benzidine to benzidine-blue which is then electively bound by the polyanions of acid mucopolysaccharides.

Introduction

Methods based on the utilization of metal-containing colloids and metallic ions play an important role in the histochemical demonstration of acid mucopolysaccharides. Such methods may be divided into several groups according to the mechanism of the procedure employed. HALE's method [5] is based on that colloidal iron hydroxide bound by mucopolysaccharides, if treated with potassium ferrocyanide, gives the Prussian-blue reaction. By employing two kinds of colloids (iron hydroxide and gold), WOLMAN [15] obtained different colour reactions for the demonstration of different mucopolysaccharides. This method was further developed by FÖLDES [4]. STEMPIEN [11] demonstrated barium ions, electively bound by acid mucopolysaccharides, by sodium rhodizonate. The nickel-dimethylglyoxime reaction employed by the present authors [12], is based on a similar principle. The same authors [13] have used the blocking effect of ferric ions for the elective demonstration of acid mucopolysaccharides by basic dyes. SCOTT [9] applies different concentrations of metallic ions in order to differentiate the various kinds of acid mucopolysaccharides.

The two methods described below are likewise based on the utilization of metallic ions, although their principle is different from that of the methods referred to in the foregoing. The first method utilizes the ability of the molybdate ions of phosphomolybdic acid to turn benzidine directly into benzidine-blue by oxidation, while it is reduced to molybdenum-blue. In the second

method metallic ions (e.g., Fe^{+++}, Cu^{++}) act as catalyzers of the oxidation of benzidine in the presence of hydrogen peroxide into a coloured reaction product.

Material and method

Samples of laryngeal and costal cartilage, muciparous glands and small intestine from white rats were used. The samples were fixed in Carnoy's fluid and embedded in paraffin. Sections of 4 to 6 μ and also cryostat sections of 6 to 8 μ were prepared.

Molybdate reduction

(1) The paraffin sections were brought to distilled water,
(2) treated with 5% aqueous solution of phosphomolybdic acid for 30 minutes,
(3) rinsed with distilled water,
(4) treated with the saturated aqueous solution of benzidine (without hydrogen peroxide) for 10 minutes,
(5) rinsed with dist. water.
(6) dehydrated and mounted as usual.

The electivity of the method was found to increase if, before treatment with phosphomolybdic acid, the sections were placed in a 5% solution of iron alum and washed, and, if after treatment with benzidine, they were placed for 5 seconds in a 0.5% solution of ammonium sulphide.

The treatment of cryostat sections began with the second step.

Benzidine oxidation

(1) The paraffin sections were brought to water,
(2) treated with a 5% aqueous solution of iron alum for 30 to 60 min.
(3) rinsed with distilled water in two steps, 2 minutes each,
(4) treated with a peroxide-containing aqueous solution of benzidine for 10 minutes,
(5) rinsed with distilled water,
(6) quickly dehydrated by tertiary butanol,
(7) treated with xylene and mounted.

Preparation of benzidine solution: dissolve 0.2 g of benzidine in 100 ml of distilled water and add 0.5 ml of 30% hydrogen peroxide. Only fresh solutions should be used.

The following salts can be substituted for iron alum: $CuSO_4$, $Co(NO_3)_2$, $Pb(NO_3)_2$. These salts, too, have to be employed in 5% aqueous solutions to obtain the desired result.

With a view to a more reliable location of the reactions the following manipulations were carried out.

(1) Methylation with hydrochloric acid—methanol at 60° C for six hours [8].

(2) Blocking of polyanions by a 1% aqueous solution of cetyl pyridinium bromide (CPB) at 30° C for 24 hours.

(3) Digestion by the aqueous solution of testicle hyaluronidase (2 mg/ml) at 37° C for 24 hours [10].

(4) Digestion by a 0.2% aqueous solution of papain (pH = 6.5) at 55° C for 4 hours [3].

Results

Molybdate reduction. As a result of molybdate reduction — performed with benzidine after pretreatment with phosphomolybdenic acid — the cartilage, the mast cells of the tissues, the muciparous glands and the goblet cells of the small intestine assumed a blue colour. The collagenous fibres and other tissue elements became pale green. The latter structures became pale brown,

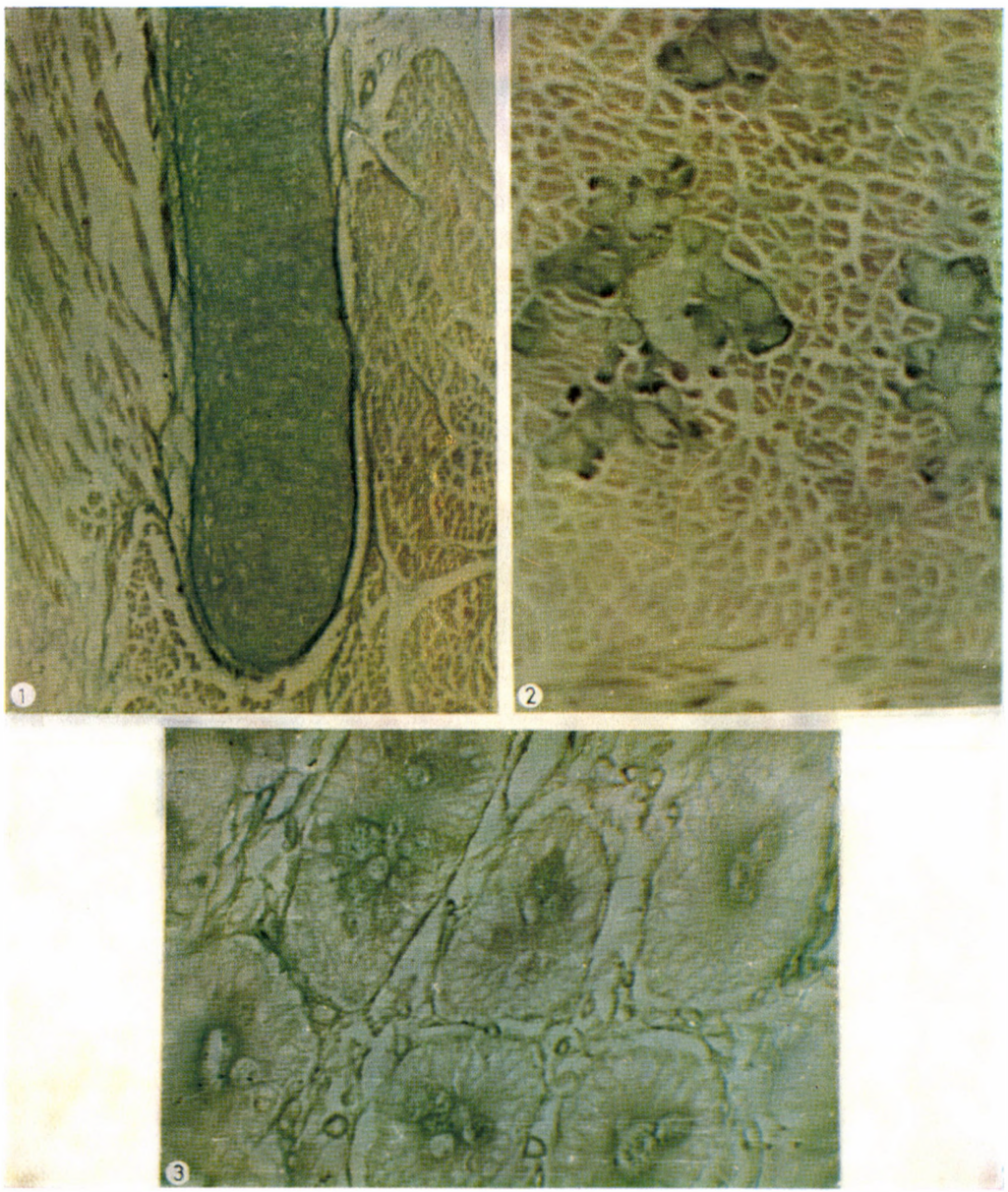

Fig. 1. Section of rat larynx. Molybdate-reduction method. ×100
Fig. 2. Muciparous glands from the root of rat tongue. Molybdate-reduction method. ×100
Fig. 3. Section of small intestine of rat. Lieberkühn crypts. Molybdate-reduction method. ×200

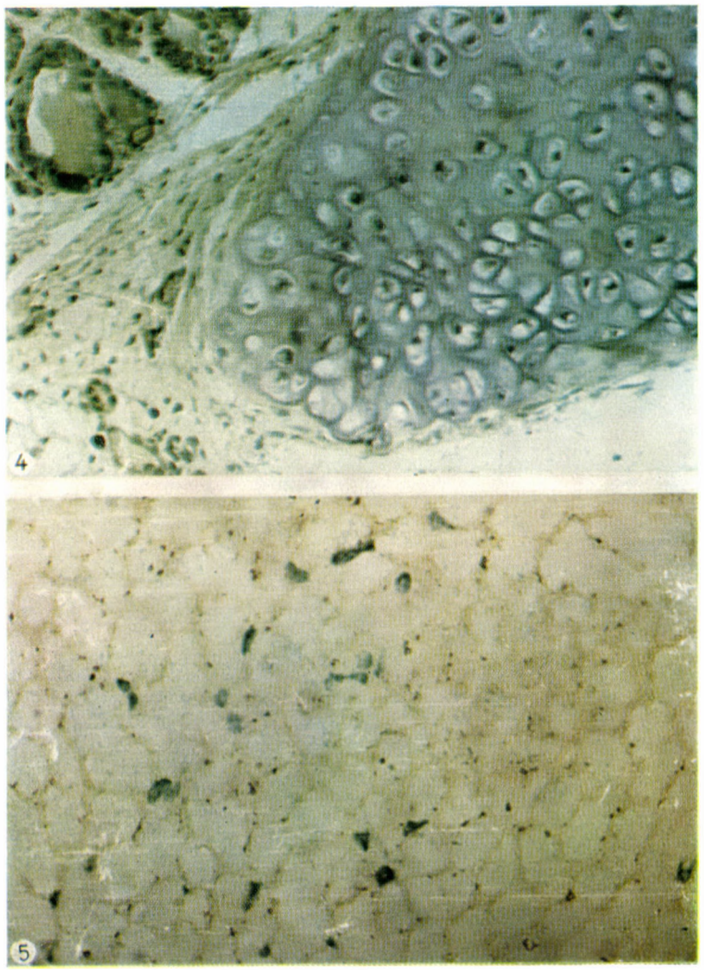

Fig. 4. Section of costal cartilage of rat. Benzidine-oxidation method. ×200
Fig. 5. Mast cells in the lymph node of rat. Benzidine-oxidation method. ×400

and the electivity was thus increased, in sections pretreated with iron alum (Figs 1, 2, 3). Advance of time did not change the blue colouring of the acid mucopolysaccharide-containing structures significantly.

Hyaluronidase digestion did not affect the molybdenum-binding capacity of the cartilaginous ground substance; the intensity of the reaction remained unchanged. The papain-digested cartilage fails to bind molybdate, it gave no colour reaction with benzidine. Methylation did not impair the intensity of reaction.

After using phosphomolybdic acid of lower concentrations (e.g. 0.1%) for pretreatment, we have observed a special reaction in some nuclei of the various tissues. This reaction demonstrates chromatin structures by a yellow-brown colour. It is possible that this chromatin-reaction is related to some nucleo-histon changes the elucidation of which needs further investigations.

Benzidine oxidation. The cartilaginous ground substance and the mast cells of the tissues stained intensively blue, while the reaction of the surrounding tissues was very weak (Figs 4, 5). The initial blue colour gradually turned brown after one or two days without a decrease in electivity. No reaction was obtained in the muciparous acini and in the intestinal goblet cells. The reactions were negative after methylation.

Discussion

Phosphomolybdic acid has long been employed for the purposes of histological differential staining. Its large anions behave like slowly diffusing colourless dye-molecules which are chiefly bound by basic proteins. Elective staining is due to competitive antagonism between the molybdate ions and the acid dye-molecules [1]. It is known that molybdate ions, when bound to tissues, are reduced to molybdenum blue when exposed to light. The highly sensitive molybdenum-blue reaction, well known in analytical chemistry, is based on the fact that — under suitable conditions — molybdenum-blue is precipitated in solutions of molybdates if treated with reducing agents. Benzidine appeared to be a suitable reducing agent for the demonstration of tissue-bound molybdate ions. In the course of this process it is oxidized to benzidine-blue while the molybdate ions are reduced to molybdenum-blue.

Colloidal molybdenum-blue has recently been used for the demonstration of structures containing acid mucopolysaccharides [4]. While details regarding the mechanism of the procedure are still obscure, it is safe to assume that the colloidal particles of molybdenum-blue are bound by the anions of acid mucopolysaccharides. In our technique, binding of the molybdate ions was followed by benzidine reduction. The question arose here, which component of the acid mucopolysaccharide complex, chondroitin sulphate or protein, was

demonstrated by this procedure. It seemed probable that the negatively charged molybdate ions were bound by the basic radicals of the protein component and not by the polyanions of the acid mucopolysaccharides. We attempted to verify this assumption by the control procedures described above. Neither blocking of the polyanions of acid mucopolysaccharides nor their digestion affected the intensity of the molybdate-reduction, whereas the reaction became negative after the removal of the protein component by digestion with papain [2]. It is, therefore, evident that the protein component of the acid mucopolysaccharides is demonstrated by our procedure.

Considering that basic benzidine-blue is also produced during the reduction of molybdate ions, it is presumable that — bound to the polyanions of acid mucopolysaccharides — it, too, plays a role in the colour reaction.

When elaborating the method of benzidine-oxidation we were guided by the well-known fact that colourless benzidine changes into benzidine-blue or benzopurpurin if oxidized by certain metallic ions, e.g., ferric, cobalt, copper, lead or gold [7, 10]. It was further known that benzidine gives the same reaction in the presence of hydrogen peroxide under the catalytic effect of the iron-containing haem- or certain iron-containing enzymes (e. g., peroxidase). WEBER's reaction and the histochemical demonstration of peroxidase are based on this phenomenon [6, 8, 10, 14].

Such considerations seemed to justify the following assumptions: (1) metallic ions are able to catalyze benzidine oxidation even if bound by tissue structures; (2) owing to its basic character the coloured product of reaction (i.e. benzidine-blue or benzopurpurin) is bound by the anions of acid mucopolysaccharides; (3) the resulting bond becomes elective — at least if ferric ions are employed. The electivity of ferric ions was demonstrated in our previous studies [12, 13].

The present experimental results have confirmed our assumptions. Metallic ions — even if bound by tissue structures — were found to catalyze benzidine oxidation. As a result, tissues containing acid mucopolysaccharides stained electively. The elective staining might be interpreted as follows. Metal ions bound by tissue structures, oxidize benzidine *in loco* to benzidine blue in the presence of hydrogen peroxide. Furthermore, ferric ions are bound to the protein component of acid mucopolysaccharides by stable bonds, while to their sulphate radicals by labile ones [12, 13]. It is therefore reasonable to suppose that iron ions catalyze benzidine oxidation irrespective of the nature of the bond; however, the basic reaction product displaces the weakly bound iron ions. Since, on the other hand, the dye-binding anions are blocked in other tissue structures by firmly bound iron ions, they are not able to bind basic benzidine dyes.

The second method described in this paper deserves interest because of its peculiar mechanism rather than on account of its practical value.

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NACHWEIS SAURER MUCOPOLYSACCHARIDE MITTELS BENZIDINREAKTION

Zwei neue Methoden für den histochemischen Nachweis saurer Mucopolysaccharide werd en mitgeteilt. Die Molybdän-Reduktionsmethode besteht darin, daß sich im Zuge der Behand lung mit Phosphormolybdänsäure die an die Protein-Komponente des sauren Mucopolysaccharid-Komplexes gebundenen Molybdationen unter der reduzierenden Wirkung des Benzidins in ein farbiges Reaktionsprodukt, in Molybdänblau umwandeln. Das Benzidinoxydationsverfahren beruht darauf, daß die an die Gewebestrukturen gebundenen Ferri-Ionen bzw. gewisse andere Metallionen in Gegenwart von Wasserstoffperoxyd die Oxydation des Benzidins zu Benzidinblau katalysieren, das sich elektiv an die Polyanionen der sauren Mucopolysaccharide bindet.

ВЫЯВЛЕНИЕ КИСЛЫХ МУКОПОЛИСАХАРИДОВ С ПОМОЩЬЮ БЕНЗИДИНОВОЙ РЕАКЦИИ

Сообщаются два новых метода для гистохимического выявления кислых мукополисахаридов. Сущность метода редукции молибдата состоит в том, что в процессе обработки фосфорномолибденовой кислотой, ионы молибдена, связанные с белковым компонентом комплексного соединения кислого мукополисахарида, под восстанавливающим действием бензидина превращаются в цветный продукт реакции, в молибденовый синий. Метод окисления бензидина основывается на том, что связанные с тканевыми структурами ионы железа, а также ионы определенных других металлов в присутствии перекиси водорода катализируют окисление бензидина в бензидиновый синий, избирательно связывающийся с полианионами кислых мукополисахаридов.

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RECENSIONES

Biochemistry of Bacterial Growth

Ed. by JOEL MANDELSTAM and K. MCQUILLEN. Blackwell Scientific Publications, Oxford and Edinburgh (1968), pp. X + 540, 84s.

This work involves the biochemical description of structures and phenomena of "generalized bacterial cell". The omission of historical aspects made it possible to systematize the subject according to the internal characteristics of the material. The excellent illustrations and tables form functional parts of the explanations detailed in the text.

Part I as emphasized by K. E. COOPER (*Nature, 220*, 624, (1968)) provides an elementary course which should prevent beginners from getting lost in the details of Part II. In the 1st and 2nd chapters of Part II the morphological structures and the main features of growth are drawn up at populational and single cell level. The energy-providing exergonic pathways (class I reactions) and the endergonic, basic component-synthesizing ones (class II reactions) are summarized in the 3rd and 4th chapters. The separate description of nucleic acid and protein metabolism (class III reactions) comprise the 5th and 6th chapters. In Chapters 7 and 8 the basic problems of genetics and co-ordination are explained while the last chapter discusses the subject of sporulation.

There are many problems in existence concerning the biochemistry of bacterial growth which go beyond the limits of the work reviewed. In spite of this, some quantum mechanical questions of nucleic acids and the significance of their methylation at the macromolecular level might have been mentioned as well.

Finally, the book is recommended because it is sure to meet the requirements outlined by the authors in the introduction.

GY. BERENCSI (Budapest)

CUSHING, D. H.: **Fisheries Biology**

A Study in Population Dynamics.

University of Wisconsin Press, Madison — Milwaukee — London (1968), pp. 200, 71s 3d.

The book of Dr. CUSHING sums up the principal current methods for the measurement of the population of economically important sea fishes; for the comparison of growth indices; for the principal habitats, migration, reproduction and alimentation of the important species. The author describes the various techniques of fishing as well as the mathematical formulae and methods of computation for the statistical evaluation of the haul. Separate chapters are devoted to the fundamental trends of piscatorial research work and to the characteristic hydrographical features of the oceans. Factors are pointed out that may inhibit a further growth of the sea-fish stock. It is in a highly instructive manner — by means of maps — that factors influencing the migration of fishes are explained, for instance, sea currents which determine the course of migration followed by the European and the American eel.

The work concludes with the explanation of the abbreviations and mathematical formulae contained in the text. The abundant bibliographical list refers mainly to the works of American and British authors. This list is followed by explanatory notes to the 82 illustrations, further by an index.

Dr. CUSHING's book made a very timely appearance, considering that public attention is nowadays directed (let us refer to the efforts of the FAO) towards the protein basis of the seas as also towards the still unexploited possibilities of high-sea fishing.

Research workers engaged in fishery, zoologists as well as economists concerned with public food supply may derive great benefit from the work under review.

B. PÉNZES (Budapest)

KIRK, R. L.: **The Haptoglobin Groups in Man**

S. Karger, Basel (1968), pp. 77, sFr/DM 20.—

The book is edited as the 4th volume of the Series of Monographs in Human Genetics. Accordingly, five of its seven chapters are dealing with the genetics of haptoglobins.

Haptoglobins are haemoglobin binding proteins of the human serum and their thorough investigation was begun in 1955, when O. SMITHIES discovered that sera from normal adults could be divided into three groups independent of the ABO or Rh blood types.

The first chapter summarizes the characteristics and properties of the three common haptoglobin phenotypes, and of their subtypes obtained by reductive cleavage. It also introduces the unequal crossing over hypothesis as the generating mechanism of Hp-2 gene.

In the second chapter the rare haptoglobin groups are listed. Data on their electrophoretic mobilities, subtyping and the possible genetic events leading to the mutants are also given.

The third chapter deals with the formal genetics of the haptoglobin groups. The two-allele model, which appears to be adequate to explain the inheritance, the three-allele model as a modified hypothesis of the former, are presented, accompanied by many illustrating examples. In this chapter, the forensic applications of the haptoglobin groups are also discussed.

The fourth chapter gives a view into the problem of the two phenotypes (Hp-2-1 mod, and Hp 0). There is still no entirely satisfactory genetic hypothesis for their control. The next two chapters cover the physico-chemical studies, the immunological reactions, the properties of the Hp–Hb complex and the formation and function of haptoglobin. The last section of the book gives an attractive population genetic study on the world distribution of the haptoglobin groups.

The book contains all the important information about haptoglobins available today. It is very useful not only for geneticists, but for scientists interested in other related fields such as blood protein research or isoserology.

ZS. HOLLÁN (Budapest)

MATTHEWS, G. V. T.: **Bird Navigation**

Second Edition, *Cambridge Monographs in Experimental Biology, No. 3*
Cambridge University Press, Cambridge (1968), pp. X + 197, 40 figs, 2 tables, £2.

The author is the most eminent investigator of bird navigation, and it is not surprising that the first edition (1955) of his work got out of print. The rapid progress of investigations have made many statements of the first edition obsolete in this short period of time. This is a completely revised edition.

Although it was repeatedly believed that problems connected with bird navigation had been definitively solved, new investigations have revealed previous conclusions to be erroneous. This incertitude is reflected by most of the titles of the book's thirteen chapters in which earlier conclusions are referred to as mere hypotheses.

It is explained that errors may originate from a lax interpretation of technical terms. Navigation, e.g., is not equivalent either to pilotage or orientation. The author presents a precise definition of the term "navigation" but fails to do so in respect of the other two terms. Since technical terms, the precise usage of which is essential, are repeatedly employed in the work, it might have been expedient to add a concise "dictionary" to the book.

It is explained in chapter I that the many-sidedness of current investigations makes it nowadays impossible to write an overall work on the migration of birds so that the present monograph deals with a special problem only, namely, navigation.

Up-to-date technical devices have promoted the study of migrations. The introduction of ringing revealed the site of distant hibernacles, proved the attachment of old birds to their accustomed places, etc. (The author omits to refer in this respect to SCHENK's pioneer work, C.R.X. Int. Zool. Congr.) Another new method was the use of parabolic reflectors for the study of flights which, imperceptible to the eye on account of their altitude, are followed acoustically; a further new device was the employment of radars, etc. All these examinations, tests and observations pointed to the fact of navigation.

Chapter II describes investigations aimed at deciding whether adhering to the route of migration is due to inheritance or acquirement (e.g., transplantation of storks; experiments with trained carrier pigeons; other experiments with mallards, etc.).

Chapter III describes experiments carried out with caged birds. These seem to show that the sun's azimuth plays a decisive role in making the birds follow the correct route.

In Chapter IV, the author deals with birds migrating at night. While admitting that the experiments carried out until now are insufficient, they seem nevertheless to show that the position of the sun when it is setting may play a primary role in navigation and that the position of the stars and the moon are of secondary importance only.

Chapter V discusses the so-called homing experiments, a matter still more intricate than the problem regarding the observation of the correct route of migration. Experiments in this respect are of two kinds: (a) some experiments were made with birds (e.g., granivorous birds, gulls, hawks, ducks, etc.) captured at the time of migration and then released elsewhere; (b) the objects of other experiments were birds captured in the nest while hatching. Especially suitable for this purpose proved the *Procellaria puffinus*, a cavity dweller, whose eggs can be prevented from cooling and the nest protected against beasts of prey in its absence. The author presents a table containing 26 species. The record is held by a specimen of *Dionedes immutabilis* which returned to its nest in 32 days from a distance of 4,000 miles. Suitable weather, good quality of the stock and release *en masse* seem to be factors favourable to the homing of pigeons. Results achieved with migratory birds were better than those obtained from experiments with non-migratory ones, especially if they were captured on the fifth day of brooding. In spite of the improving facilities, the experiments are desired.

It is explained in Chapter VI that the experiments instituted in order to ascertain whether the birds immediately find the correct course are likewise insufficient. A long series of experiments seems to show that this is so, but, within 20 miles, also a sense of locality appears to be operative. The experiments have the limitation that — at least for the time being — the time of observation lasts a few seconds only.

Chapter VII discusses hypotheses concerning the probable connection between the bird and its home (e.g. vision, physical and chemical perception of the atmosphere by means of air pockets, nasal glands, radiation, etc.).

The conclusion reached by the author in Chapter VIII is that the numerous and many-sided investigations regarding the Corioli force, magnetism, emission of short waves, climatic conditions, etc., have so far failed to yield satisfactory results.

Chapter IX discusses the current theories of navigation and concludes that the majority of arguments speaks approving that navigation follows the sun.

Chapter X holds out hopes for promising results stating that the co-ordinates of the sun are decisive factors of navigation. Chapter XI proves this statement. It is emphasized that birds are attached to the place where they achieved sexual maturity and not to their place of hatching. The author describes the heated debate raging about this question.

On the analogy of other animals (flies, etc.), in Chapter XII the author points out that anatomical and physiological examinations of the bird eye support astronavigation.

The last chapter discusses the question as to how the vision of birds is affected by their movements; it deals further with the birds' sense of time and the role of their memory.

The book contains an abundant bibliographical list and a concise but very useful index.

The monograph is undoubtedly a valuable addition to the literature of biology.

A. KEVE (Budapest)

MÜLLER, E. and LOEFFLER, W.: **Mykologie**

Thieme, Stuttgart (1968), pp. 302, 170 illustrations, DM 9.80

The general part of the book covers 102 pages, while 170 pages are devoted to applied mycology. The bibliographical list and the index occupy the remaining 30 pages. The book is provided with a tastefully executed synthetic jacket the inner side of which contains instructive data regarding diseases caused by poisonous fungi.

The general part begins with the determination of the place of fungi among the living organisms. Orientation in this respect is facilitated by a table. The order of Actinomycetales is classified under the head of bacteria. The essential requirements and biological properties of fungi are discussed in the next chapters which are followed by fundamental information about morphology, fungal colonies, hyphae and reproductive organs. The physiological part of the work deals with the DNA- and RNA-activity of fungi, their protein and carbohydrate metabolism in accordance with up-to-date theories. Orientation is facilitated by chemical formulae and illustrations.

Utilization of fungi is the subject of the opening part of Applied Mycology. It offers a concise and good survey on the use of edible fungi and the role played by useful fungi in the manufacture of alcoholic beverages, fermented foodstuffs, bread and cheese as also in the economical large-scale production of chemical compounds. The next chapters deal with the noxious effects of fungi on fruits, foodstuffs, woodenware, leather goods and textiles. Information regarding mycotic diseases is summed up on five pages. It is emphasized that the view that fungi have only metabolic products but no toxins is no longer valid. *Aspergillus flavus*, *Stachybotrys atra*, *Penicillium* and *Fusarium* do produce toxins. — Though in proportion with the size of the book, the medico-veterinary part of the work is regrettably short.

The class names Archimycetes and Phycomycetes have been left out the taxonomy to be replaced by a detailed classification ranging from the Oomycetes to the Zygomycetes. Ascomycetes are divided into the groups of Euascomycetes and Endomycetes. That the latter are universally accepted as corresponding to the Hemiascomycetes is not indicated by the authors, although it might have been advisable to treat them as synonyms. There are numerous forms of ascus production, and the characteristic ones are treated in detail in the part on true Ascomycetes. A key is given in this connection. The symbol 1 of drawing No. 93 on page 183 points to the hymenium and not to the wall of the fruit body.

The chapter Basidiomycetes is followed by that on the Fungi imperfecti. Page 264 contains the well-known classification based on the forms of reproduction, viz. Moniliales, Sphaeropsidales, Melanconiales. It is stated in the last lines of the chapter Moniliales that acervulus and sporodochium are characteristic of the Tuberculariaceae, a statement rejected by other authors. Acervulus characterizes, according to FUNDER, WOLF and WOLF, the conidium production of Melanconiales, whereas widened and modified hyphal forms, serving for the formation of conidia, are as characteristic of Tuberculariaceae as is the formation of coremium among the Stilbaceae.

To enumerate the spore types of the Fungi imperfecti seems to be somewhat unnecessary since conidia are dealt with on page 53. On pages 266—277, the various kinds of spore are discussed anew, e.g. annelospore, radulaspore and porospore, although they are all conidia. The aleurispores are compared to the chlamydospores, although, according to the dominant theory, aleurispores represent conidia which start an independent life only after the atrophy of the hyphae. It would have been preferable to deal with these asexual spores together with the conidium and macroconidium. The number of penicillium species is stated to total 150 (paragraph 2 on page 271), whereas, in reality, it exceeds 200.

In summary, the book offers — in proportion to its size — sufficient information on mycology. The chapters dealing with the morphology, biology and genetics of fungi may be of interest also for physicians, although medico-mycologists will derive more benefit from them. The chapter discussing the characteristic symptoms and the pathogens of mycoses for the benefit of general practitioners and dermatologists is somewhat too concise which is undoubtedly due to the general size of the book.

E. FLÓRIÁN (Budapest)

PALKOVITS, M. and FISCHER, J.: **Karyometric investigations**
Akadémiai Kiadó, Budapest (1968), pp. 347

There is an increasing body of evidence indicating that changes in functional activity of the cell lead to changes in nuclear size. Swelling of the nucleus means usually an enhanced function, shrinkage a diminished cell activity. It has been shown that nuclear size changes occur very rapidly (within less than an hour), and that there is a linear relationship between non-histonic protein contents and size of the nucleus. Since nuclear size appears to be a fairly sensitive indicator of changes in functional activity of the cell, the number of karyometric investigations increased considerably in the last years.

This book is the first attempt to summarize the various aspects of karyometry. The authors are experts in this field, who made several important contributions.

After an introduction, the theoretical aspects of nuclear size measurement are discussed, dealing with cell division, functional activity of the cell, regeneration, pathological factors, sex differences, diurnal and seasonal rhythm and age, all which affect nuclear volume. Next chapter is concerned with the techniques of measuring and computing nuclear volumes. Direct microscopic measurements, microprojection methods, automatic and semiautomatic measuring instruments, and statistical evaluation of nuclear volumes are described here. Chapter IV gives a complete and detailed analysis of the mathematical aspects of karyometry (statistical

tests, effect of magnification, influence of shape and spatial arrangement of nuclei, theoretical models and accuracy of calculations, etc.). A brief chapter deals with the measurements of nucleolar volume. The effect of fixation, embedding and various stains on nuclear size, and the necessary number of measurements are discussed in a separate chapter. About a third of the book is devoted to "systematic karyometry". In this part the karyometric data of all tissues and organs of the organism are summarized and discussed. A vast amount of the authors' own material is presented in this section. Bibliography contains about 1000 references. Tables necessary for statistical evaluation of the nuclear measurements are attached to the book.

The book is well produced and the subject matter clearly presented. It contains much useful and stimulating information which will be of interest to anyone interested in the quantitative and fairly sensitive morphological method of karyometry. The book is especially recommended for those who work with this method.

B. HALÁSZ (Pécs)

WALSH, E. O'F.: **An Introduction to Biochemistry**

2nd edition. The English University Press Ltd., London (1968), pp. 482, 45s.

Students and especially medical students badly require a short introductory course on contemporary biochemistry. Existing texts fail mostly because of undue emphasis on chemical details and offering not much insight into the contemporary concepts of biochemistry. Professor WALSH sets out to "introduce the student to biochemistry as a natural philosophy" as distinct from "science" dominated by technology. This is a most laudable project. Unfortunately, he fails to do so and provides the student with an outdated and uninspiring book. Many authors are aware of the difficulty of bringing out a textbook of biochemistry and keep it up-to-date during succeeding editions. Professor WALSH has written this text apparently 15 years ago, and revised some parts about 7 years ago. With the rapid advance of research our understanding has changed too much during these years to admit such a procedure. Biochemistry, as part of biology, has produced a wealth of data concerning the relation of molecular structure and biological activity, and these data have led to new concepts enriching "natural philosophy". Organized enzyme systems, regulation of metabolic processes through allosteric mechanisms and through repression, membrane transport phenomena, immune reactions, the molecular mechanism of muscle contraction and nerve conduction, the biochemical architecture and organization of the cell, etc. are subjects on which a student should obtain a generalized picture and up-to-date information. The present paperback edition sadly fails in providing these fascinating subjects and sometimes fails even to the extent of giving wrong impression (e.g. on enzymatic adaptation, Chapter 13). This book is definitely not recommended to students.

F. B. STRAUB (Budapest)

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COMPARATIVE HISTOCHEMICAL INVESTIGATIONS CONCERNING PRENATAL AND POSTNATAL CHOLINESTERASE ACTIVITY IN THE HEART OF CHICKENS AND RATS

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(Received April 29, 1968)

Synopsis

Studied by histochemical methods in chick and rat embryos and newborn and adult rats, cholinesterase activity was detected in the myocardial cells before the vagus nerve has reached the heart. Soon after vagus innervation the enzyme revealed intense activity as the heart developed, which declined gradually. The day-to-day decrements were unimpressive but the loss over ranges of several days was obvious; particularly in the embryonic rat heart. Decline of activity and differentiation of heart cells follows a similar course.

Introduction

Cholinesterase activity in the heart of adult animals is well documented. Using his own histochemical method, GOMORI [6] found this activity was intense in the zone of the conductive system. RAVIN and co-workers [16] localized it also in the myocardium. Working with cattle, guinea-pig and rabbit cardiac muscle, and employing the method of KOELLE [11], DUMONT and DROUIN [5] detected activity only in neural elements closely connected with conducting fibres. Confirming this finding, MOHR and GEREBTZOFF [14] claimed that the sarcolemma of conducting fibres, too, gave positive cholinesterase reaction, but SCHIEBLER [17, 18] denied this claim. CARBONELL [2] was the first to insist that in the mammalian hearts he studied, this activity was restricted to the myofibrils of the conducting fibres. ÁBRAHÁM and ERDÉLYI [1] who studied the sino-atrial and the atrioventricular nodes in ungulates, and JAMES and SPENCE [8] who studied them in humans, produced conclusive evidence that, in addition to the strongly positive nerve fibres and ganglia, the conductive system also shows positive reaction with cholinesterase, and that in humans, even the fibres of the right atrium are positive for this enzyme.

Two points seem not to have been raised before in the literature: can cholinesterase activity be demonstrated in the embryonic heart by histological methods, and if so, can such methods inform about changes that may take place in this activity during ontogenesis? The present experiments were designed to find the answers to these questions.

Material and method

Hearts from Leghorn chicks between the third day of incubation and the first day after hatching, and hearts from laboratory white rats between the 10th day of intrauterine life and the 21st postnatal day, were used in these experiments. Chicks of less than 6-day incubation, and rats of less than 14 days of intrauterine life, were fixed in 4 per cent formol-Ca in the whole, with the hearts *in situ*. From animals at later stages of growth, the hearts were removed and fixed *in toto*. In their case, fixation times had to be adjusted as the myocardia grew gradually thicker; they had to be long enough to fix the soluble enzyme *in loco*, but not so long as to reduce actual enzymic activity significantly. Accordingly, chick hearts until the tenth day of incubation and rat hearts until birth, were fixed for five hours. Older hearts were flushed several times with fixative through the atrium and fixed for 12 hours. The ventricles and atria were then carefully cut open to ensure that the fixative should permeate the heart-muscle fibres simultaneously from the epicardium and the endocardium. These procedures made it possible for us to compare cholinesterase activities in hearts at all developmental stages.

Sections of 15 to 20 μ thickness were cut serially in a cryostat, and always in the frontal plane to enable reconstruction of the sino-atrial, atrioventricular, and bulboventricular areas, of the developing Aschoff-Tawara node, the bundle of His, and the Purkinje fibres, with a view to studying them for cholinesterase activities. Activities were demonstrated by the method of KARNOVSKY and ROOTS [10]. Acetylthiocholine and butyrylthiocholine iodide served as substrates. Control sections were treated with an incubation medium containing 10^{-5} *M* eserine. The incubation time was 2 hours at 37°C, pH 6.2. The two substrates yielded approximately the same results.

Results

Embryonic chick heart

Three-day and four-day hearts. The wall of the ventricle and the atrium consists of one or two cell layers. No septum is present. The heart is elongated on the third, and perceptibly sigmoid on the fourth day. Cholinesterase activity is seen in the muscle fibres of the wall of the veins which drain into the sinus venosus and in those building up the atrioventricular wall; in the latter, it is more impressive.

Five-day and six-day hearts. The wall of the ventricle begins to thicken, but that of the atrium remains thin. Interlacing, the ventricular fibres form a trabeculate structure. Cholinesterase activity is low in the ventricular and atrial fibres, comparatively higher in the muscle rings, particularly in the one in the atrioventricular area (Fig. 1). The primordial ventricular septum appears at the end of the sixth day; in the group of cells from which it is to develop enzymic activity is intense (Fig. 2).

Seven-day and eight-day hearts. The ventricular fibres, particularly those subjacent to the epicardium, become closely packed. The trabecular structure disappears, and the wall of the ventricle grows more compact. The atrial wall is still thinner than the ventricular. Cholinesterase activity is intense both in the ventricular and in the atrial fibres. The centrally placed cells of the developing ventricular septum are much more active to the enzyme than are those at the periphery. In the area of the atria above the ventricular septum a loose group of muscles of intense enzymic activity, makes its first appearance. The fibres of these muscles presumably represent the anlage of the future atrial

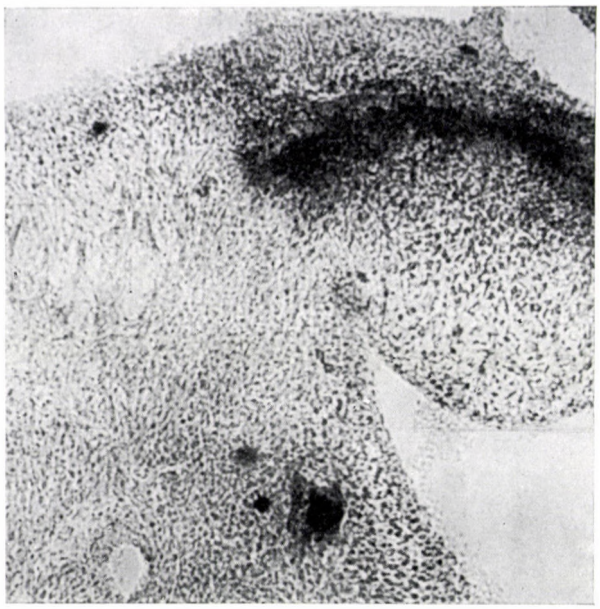

Fig. 1. Cholinesterase activity in the atrioventricular muscle ring of a 7-day-old chick embryo. Karnovsky's acetylthiocholine iodide. ×480

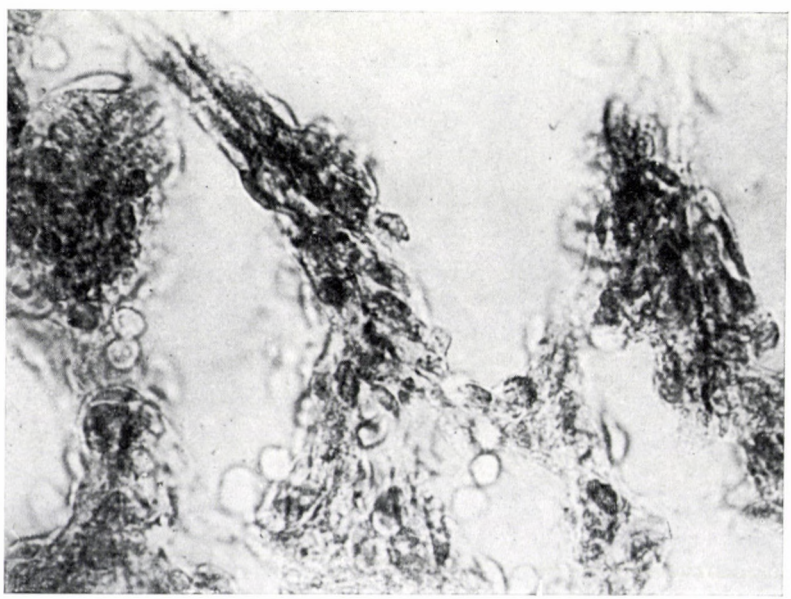

Fig. 2. Cholinesterase activity in the developing ventricular septum of the heart of a 7-day-old chick embryo. Karnovsky's butyrylthiocholine iodide. S = septum. ×480

septum. The anlage for the bundle of His also becomes visible. It is more active to cholinesterase than are the ventricular fibres adjacent to it. Cholinesterase-positive nerve fibres appear in the pacemaker area and in the epicardium between the atria and the ventricles. Then and when a section shows slightly higher activity in the subendocardial than in the subepicardial fibres.

Nine-day and 13-day hearts. In this period, cholinesterase activity declines in the ventricular fibres but remains unchanged in the Tawara-His node, and so the conducting system can readily be distinguished from muscle fibres. The



Fig. 3. Cholinesterase activity in the atrium and ventricle of a 15-day-old chick embryo-Karnovsky's acetylthiocholine iodide. ×200

ventricular septum and the ventricular fibres are now of the same activity. The reaction in the muscle fibres above the ventricular septum grows stronger. The positive nerve fibres of the heart increase in number. Cholinesterase activity is higher in the fibres of the right than of the left ventricle. In the atrial fibres it is as intense as in the, by now, fully developed atrial septum.

Fourteen-day and 15-day hearts. In the outer ventricular wall muscle fibres characteristic of the adult chick-heart make their first appearance. Trabecular fibres are seen only as a thin layer in close proximity to the endocardium. Cholinesterase activity continues to decline in the ventricular fibres but remains intense in the atrial fibres (Fig. 3). This difference continues to the end of the embryonic life and, in some measure, is detectable in the adult chick heart. Situated mostly in the upper part of the ventricular septum and among muscle fibres in the right ventricle, the thin terminal ends of the intracardiac

nerve fibres begin to show in faint outline. The reaction is strong in the bundle of His and Tawara-His node. In this stage it is difficult to differentiate the activity in the conducting fibres proper and the surrounding nerve fibres.

Seventeen-day and 18-day hearts. Cholinesterase activity continues to decline in the ventricular muscle fibres but remains unchanged in the atrial fibres. It is highly conspicuous in the sino-atrial node. The terminal nerve fibres in the ventricular area show maximum activity for cholinesterase.

Hearts in the period from the 19th day to hatching. Light microscopic studies revealed no qualitative or quantitative changes in cholinesterase activity.

Rat heart in the embryonic period

First 16 days of gestation. No cholinesterase activity is detectable by histochemical methods before day 13, on which day it abruptly grows intense both in the ventricular and in the atrial muscle fibres. The fibres in the auricular

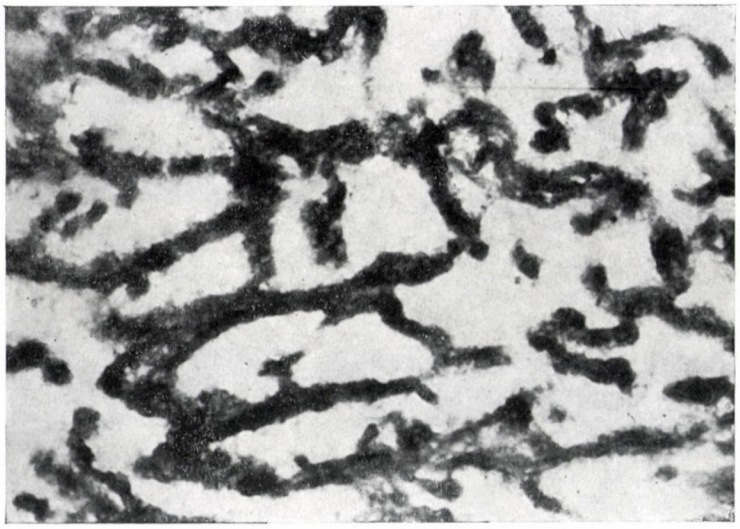

Fig. 4. Cholinesterase activity in the trabecularly structured ventricle of a 14-day-old rat embryo. Karnovsky's butyrylthiocholine iodide. ×240

ring are more active than the atrial and ventricular fibres. Conspicuously active is also the wall of the bulbus cordis. The developing ventricular septum becomes distinct at day 14 and activity in it is as high as in the ventricular fibres. The ventricular walls begin to thicken gradually, assuming trabecular pattern (Fig. 4). In this period, the fibres of the conductive system cannot be distinguished from the heart muscles on the basis of cholinesterase activity, though an occasional section may give the impression that activity is slightly lower in

the subepicardial muscle fibres of the ventricular wall than in the subendocardial fibres.

From day 17 of gestation to birth. At days 17 and 18, two layers become visible in the ventricular wall: in the outer layer the muscle fibres are closely packed, and the inner layer shows a trabecular pattern of undifferentiated fibres (Fig. 5). No similar stratification is seen in the atria. The ventricular

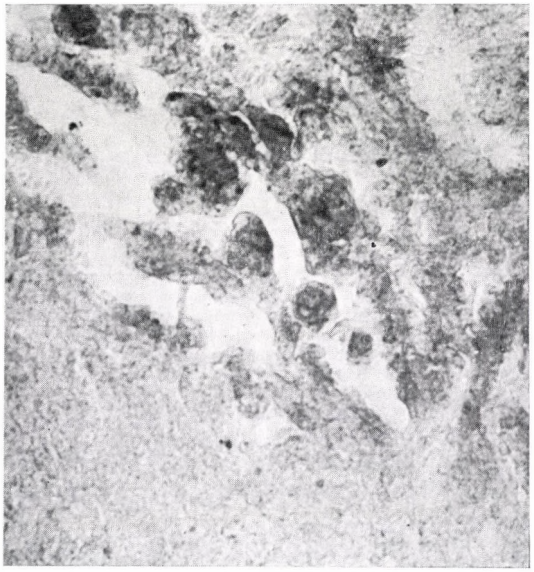

Fig. 5. Cholinesterase activity of the ventricle in the heart of an 18-day-old rat embryo. Karnovsky's butyrylthiocholine iodide. ×480

septum reaches the auricular ring, making the separation of the two ventricles complete. In its upper part, the bundle of His becomes discernible as an area differing but slightly in activity from its surroundings. Activity in the atria agrees with that seen in the inner layer of the ventricular fibres. Sporadically, cholinesterase-positive nerve fibres appear for the first time. With proceeding development, the outer layer of the ventricle grows gradually thicker and the more active inner layer of trabecular pattern becomes thinner. At the same time, the positive nerve fibres increase in number, and the bundle of His shows striking activity against the declining activity in the muscle fibres adjacent to it.

Immediately before birth, cholinesterase activity is seen to have markedly declined in the muscle fibres of the ventricle, and to have remained unchanged in those of the conductive system and the atrium. In the neural elements of the heart, it continues to increase. In the atrium, only the subendocardial fibres show high activity; they are assumably Purkinje's fibres.

Postnatal development of the rat heart. In the period immediately following birth, no essential change is observed in the cholinesterase activity of the rat

heart. Between this period and maturity, activity is noted to continue declining slightly, mostly in the ventricular fibres. Light microscopy fails to detect any change in the activity of the atrial and the conducting fibres. At the age of 21 days, conditions prevailing in the adult rat heart are seen: cholinesterase is substantially higher than in the hearts of other laboratory mammals, and is

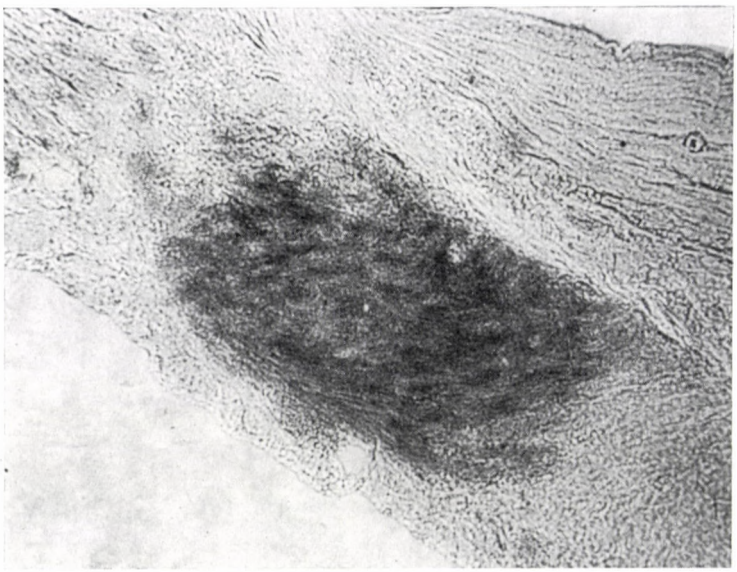

Fig. 6. Cholinesterase activity in the bundle of His of a 21-day-old rat. Karnovsky's acetylthiocholine iodide. ×800

greatly increased in the area of the conducting fibres. For the latter phenomenon the nerve fibres tightly encompassing the conductive fibres, are mainly responsible (Fig. 6).

Discussion

The present work provides evidence that to cholinesterase which is sensitive to 10^{-5} *M* eserine, the chick heart exhibits activity on the third day of incubation and the rat heart on the 13th day of gestation; i.e., long before the vagus nerve enters the heart. Soon after, but still before vagal innervation, the heart starts beating in both species, activity rapidly increases, and attains its maximum. No significant change in activity occurs at the time the vagus nerve reaches the heart. As the heart develops, this activity declines, and though the day-to-day decrements are unimpressive, the loss over ranges of several days is obvious; particularly in the embryonic rat heart. Decline of activity and differentiation of heart cells follows a similar course. Evidence of this is best seen in 16—17-day-old embryonic rat hearts: activity is lower in the outer layer of the ventricle composed of older, more differentiated cells, and

higher in the inner layer consisting of younger, less differentiated cells. In the conducting fibres, cholinesterase activity is intense throughout growth, and it becomes increasingly difficult to distinguish them from the surrounding nerve fibres by this activity.

In the embryonic chick heart the pattern of cholinesterase activities is on the whole similar to that in the embryonic rat heart, barring two observations: one is that the more differentiated ventricular layer cannot be as readily distinguished from the less differentiated layer in the chick heart as in the rat heart; the other, of course, is that the conditions characteristic of the adult heart present themselves in the chick one day after hatching and in the rat around the 21st day after birth.

Our observations tally well with data from the work of SIPPEL [23] who studied cholinesterase activity in chick and rat hearts during ontogenesis by biochemical methods.

While it is common knowledge that the less differentiated the myocardial cells are the higher is their spontaneous activity, in the present work it has been shown that the lower is their degree of differentiation the higher is their cholinesterase activity. From this it may be inferred that in the cells of the embryonic chick and rat heart a direct relationship exists between spontaneous activity and cholinesterase activity.

At this point it is inevitable that the question as to the acetylcholine content of the cells should arise.

The high cholinesterase activity in the embryonic myocardial fibres suggests that the amount of acetylcholine in the heart is substantial in the earliest developmental stages and subsequently decreases gradually, in other words, that cholinesterase activity is directly correlated with the amount of acetylcholine. Data from the work of MAZEL and HOLLAND [13] confirm this view. These authors found that at sites of high cholinesterase activity in the adult frog and turtle heart, e.g. in the sinus venosus, the acetylcholine content was also high, the latter depending on choline acetylase activity and on the quantity of acetyl coenzyme A.

Employing biochemical methods, VLK and TUCEK [24, 25], on the other hand, found that in the hearts of a variety of adult mammals the cholinesterase distribution did not correspond to the acetylcholine distribution. SINGER and co-workers [22] observed very little cholinesterase activity and remarkably great cholinesterase content in the early regenerating forelimb of the newt.

Published data on electrophysiological investigations favour the idea of inverse relationship [7, 3]. They provide evidence that acetylcholine depresses the slow diastolic depolarization which characterizes the fibres of the conducting system, and so their membrane potential becomes the same as that of the myocardial fibres not beating spontaneously; i.e., the fibres of the conducting system cease to beat. From the conception formulated by CRANEFIELD and

HOFFMAN [3] it follows that slow diastolic depolarization occurs when the permeability of the fibres is on the decline. This, in turn, suggests the theoretical conclusion that — since there exists a direct relationship between the amount of acetylcholine and the potassium-permeability of the membranes — at the time spontaneous depolarization occurs and the muscle fibres' permeability to potassium is low, acetylcholine must be small in amount. However, the histochemical studies referred to in the Introduction to this communication all tend to prove high cholinesterase activity in the fibres of the conductive system.

Regrettably, no published information is available about similar electrophysiological investigations of embryonic myocardial cells. However, DEHAAN [4] who studied their permeability to potassium in great detail, found that in the spontaneously active heart cells of the chick embryo it is low but gradually increases during ontogenesis; further, that in proportion to its increase, more and more cells cease to beat spontaneously. If there exists a straight-line correlation between the potassium permeability and the acetylcholine content of the myocardial cells, then it seems reasonable to suppose that permeability to potassium may inform about acetylcholine content. This line of approach leads to the conclusion that high cholinesterase activity in the embryonic myocardial cells is not associated with a simultaneous high acetylcholine level.

According to present-day knowledge, the ratio of bound to free acetylcholine differs in the myocardial cells of embryonic and adult animals. PETERSON and BASERGA [15] reported substantial decrease in the rate of protein synthesis in the embryonic mouse heart during ontogenesis. In the younger myocardial cells protein synthesis is high and protein content low, and in older cells it is just the other way round. This means that the protein content is the factor which limits the amount of bound acetylcholine in the heart cells.

The likely changes in the acetylcholine content of the myocardial cells require further biochemical studies.

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VERGLEICHENDE HISTOCHEMISCHE UNTERSUCHUNG DER PRÄ- UND POSTNATALEN CHOLINESTERASE-AKTIVITÄT IN HÜHNER- UND RATTENHERZEN

Mit Hilfe histochemischer Methoden wurde die Cholinesterase-Aktivität im Herzen von Hühner- und Rattenembryonen, ferner im Herzen neugeborener und älterer Ratten untersucht. In beiden Tierarten läßt sich die Cholinesterase-Aktivität in den Myokardzellen noch bevor der N. vagus hineinwächst nachweisen. Die Aktivität steigert sich zunächst rasch, um später während der Ontogenese allmählich abzusinken. In den einzelnen einander folgenden Tagen ist diese Aktivitätsverminderung nicht auffallend, doch ist sie in größeren Zeiträumen gut nachweisbar. Die Abnahme der Cholinesterase-Aktivität verläuft parallel mit der Differenzierung der Herzmuskelzellen.

СРАВНИТЕЛЬНОЕ ГИСТОХИМИЧЕСКОЕ ИЗУЧЕНИЕ ПРЕД- И ПОСЛЕРОДОВОЙ АКТИВНОСТИ ХОЛИНЭСТЕРАЗЫ В СЕРДЦЕ ЦЫПЛЯТ И КРЫС

Автор изучал с помощью гистохимического метода холинэстеразную активность в сердцах зародышей цыплят и крыс, а также в сердцах новорожденных и более старших крыс. Активность холинэстеразы можно выявить в клетках сердечной мышцы обоих видов животных еще до врастания блуждающего нерва. Холинэстеразная активность быстро усиливается, а затем, в течение онтогенеза она постепенно уменьшается. Понижение активности в отдельные последующие друг за другом дни незначительное, но оно хорошо выявляемо в более больших интервалах. Уменьшение холинэстеразной активности происходит параллельно с дифференциацией сердечной мышцы.

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THE CYTOLOGICAL INDICATORS OF ROOT GROWTH

VI. CHANGES OF RIBONUCLEASE ACTIVITY IN THE CELLS OF ROOT SEGMENTS

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Synopsis

The author examined the ribonuclease (RNase) activity in isolated 1 mm segments of maize *(Zea mays* L.) root. The activity expressed in relative units was related partly to the various segments and partly to the protein-N unit of the cell. The experimental results revealed that it is suitable to use several reference bases (segment, cell, protein-N) for the valuation of enzyme activity. The greatest RNase activity in cell populations of the different root segments was found in the zones showing intensive developmental metabolism. In segments of increasing distance from the apex and containing differentiated cells the RNase activity per segment or protein-N gradually diminishes, but grows per cell or its protein-N. Accordingly, the activity of the RNase enzyme shows a direct connection with the protein-N content of cells and their developmental intensity. It could also be demonstrated that the RNase enzyme is suitable for the characterization of differently developed cell populations and may, therefore, also be considered as the cytological index of root growth.

Introduction

In his previous works [6, 7, 8] the author has dealt with several developmental indexes manifesting themselves in cell populations of the different segments of young roots. The changes of enzyme activity are also valuated as physiological gradients of tissues and cells by literary data [1, 2, 4, 11, 15, 16, 18]. The ribonuclease (RNase) has also shown a connection with the change of metabolism and growth according to HANSON and co-workers [3], who have concluded that the senescence of tissues is caused by the alteration of the enzyme. SCHWEIGER [12] found a connection between the RNase activity and genetic information. Others, so LUND and co-workers [5] have pointed to a relation between the growth and development of root cells on the one hand and the RNase activity on the other, while in pea mesocotyl a direct correlation between the RNase activity per protein unit and the differentiation degree of cells has been observed by SHANNON and HANSON [13]. Therefore, it seemed to be justified to elucidate how the RNase activity is affected in cell populations of different root zones by the developmental stage of cells. In addition, it had to be demonstrated, whether the alteration trend of cytological indexes established previously [6, 7, 8] is followed by a change of RNase activity thus being really suitable to characterize the stages of development and differentiation in cell populations of the different apical root zones.

Material and method

As experimental material the "Mv_1" hybrid variety of maize *(Zea mays* L.) was used raising and preparing it identically with the procedures described previously [6, 7].

For measuring the RNase enzyme activity the modified method of McDonald [10], Tuve and Anfinsen [17] was applied. From equally long maize roots accurately 1 mm long discs were cut with a special device [9]. The tissue homogenizate was prepared by trituration. The incubation was carried out with 0.1% yeast RNA dissolved in 0.1 *M* acetate buffer of 5.0 pH at 37°C for 30 minutes. The reaction was interrupted with the McFadyen reagent; subsequently the mixture was centrifuged and the optical density of the supernatant established after tenfold dilution. For measuring at 260 mμ an "Unicam" spectrophotometer was used. One enzyme unit equals with an 0.10 increase of optical density above the control precipitated at the 0 moment. The mutual percentile proportion of RNase activity in the different segments as well as its rates per protein-N and per cell were also established. The statistical valuation was performed according to Snedecor [14].

Results

The experimental results are presented in Table 1 and Fig. 1.

Table 1

Relative RNase activity in isolated apical segments of maize root
($\bar{x}$ = mean; s = standard deviation; CV = coefficient of variation)

Distance from the apex	RNase units per segment				RNase units per μg protein-N	RNase units per cell	
mm	$\bar{x}$	s	CV	%*		10^4	
1	15.4	1.5	9.7	20	3.7	1.94	(5%)*
2	45.6	8.1	17.7	61	10.3	6.50	(17%)
3	74.6	3.9	5.2	100	18.8	19.63	(51%)
4	65.9	10.6	16.0	88	17.8	19.36	(50%)
5	25.1	1.0	3.9	34	8.3	16.73	(43%)
6	30.0	2.0	6.6	40	7.3	27.27	(70%)
7	35.1	7.4	21.0	47	11.0	36.36	(95%)
8	32.7	7.2	22.0	44	10.4	38.47	(100%)

* The highest value = 100

The values of the relative RNase activity measured in the various segments are very different: the initial degree attains a fivefold increase from the apical segment to the third one and subsequently the maximum value diminishes by about 50 per cent. The greatest activity may be found in the third and fourth segments showing intensive cell expansion and substance augmentation. This establishment is especially well demonstrated by the percentual values (Table 1 and Fig. 1).

The values per protein-N unit resulted in a curve of similar course as that of the data per segment. From this fact the conclusion can be drawn that

with increasing distance from the apex the quantity of protein-N diminishes in the segments, as it was also established in other papers of the author.

The RNase enzyme activity of cell populations found in the various segments and related to one cell shows quite another picture. Essentially, with increasing distance from the apex the activity per cell gradually runs up, as it is also demonstrated by the percentual values. This means that the activity of

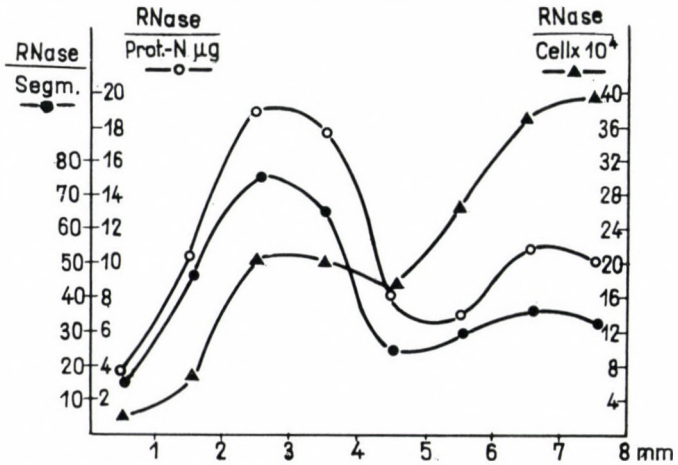

Fig. 1. Changes of RNase enzyme in unlikely differentiated cell populations of apical root segments of maize.
Ordinate: RNase activity (arbitrary unit)
Abscissa: Distance from the apex

RNase enzyme increases collaterally with the differentiation degree of cells in the examined root piece, and between the weight as well as the size of the cells and their nucleic acid and protein content — rising also with increasing distance from the apex — a connection may be supposed.

Discussion

On the valuation of enzyme changes in plant tissues as physiological indexes many data have been presented by literature. So certain relations were found: between the activity of proteinase and the change of cell volume by BROWN [1]; between the decrease of deoxyribonuclease and the division rhythm of root cells by GOUTIER and GOUTIER-PIROTTE [2]; between the invertase and cell expansion by HELLEBUST and FORWARD [4]. Close connections have been evidenced: between the cytochromoxydase and the growth metabolism of root cells by POTAPOV and SALAMANTOVA [11]; between the peroxydase activity of root cells and the degree of their differentiation by SMIRNOV and co-workers [15]; between the changing activity of the various enzymes and the develop-

mental rhythm of cells by TORREY [16] and WARDLAW [18]. Accordingly, the changes of enzymes may demonstrate the metabolic alterations and the developmental stage of cells.

The change of RNase activity may also be considered as a characteristic of the metabolic state in plant cells. It has been done so by SCHWEIGER [12] concluding from the RNase activity of *Acetabularia* to the transmission of genetic information. HANSON and co-workers [3] have found that the connection between the respiratory intensity of maize endosperm and its RNase activity expresses the senescence of cells. SHANNON and HANSON [13] have evidenced that a close correlation exists between the per-cell protein quantity of the pea mesocotyl and its RNase activity. The RNase activity determined for the protein unit of the cell increases namely from the zone of undifferentiated cells toward the mature ones. Simultaneously and parallel with the degree of differentiation also the quantity of the protein in the cells grows larger, while the amount of ribonucleic acid diminishes. LUND and co-workers [5] have pointed out that the growth and development of maize root cells are connected with the changes of RNase activity. So it has been proved by literary data that the RNase activity of plant cells generally increases according to their growth and ripening. However, due to differences partly in test plants and partly in the bases of reference any comparison is very difficult.

In the course of the experiments reported here connections were found between the developmental stage of cell populations in various segments of the root apex on the one hand, and the protein-N and nucleic acid content as well as the respiratory intensity and catalase activity of cells on the other [6, 7, 8].

Therefore, it was supposed that by the RNase activity — being in close connection with the protein synthesis, i.e. with cell growth and devolopment — the differentiation degree of root cell populations is also clearly reflected; the experimental results confirmed this assumption. Starting from the apex, the RNase activity per segment gradually increases, but progressively diminishes later. It is, accordingly, low in the zones of still dividing and already differentiated cells, but shows a high value in the zones of greatest cell expansion. The activity data per protein-N unit of the various segments yield a similar curve. This is due to the fact that in segments containing cell populations in which the division has already ceased, the protein-N content gradually decreases toward the root base. On the other hand, the RNase activity per cell in the various segments runs up with moving off from the meristematic dividing cells of the root apex and in accordance with their degree of ripening. The highest values are shown by the zones of entirely differentiated cells. The apparent contradiction between the values per protein-N and those per cell derives from the fact that though the zones of differentiated cell populations contain less protein, relatively few cells can be found in them just due to their large size and entirely differentiated state. Therefore, their protein-N content is also larger

than that of those in segments near to the apex. This establishment corresponds well with the data of SHANNON and HANSON [13] on pea.

The results accordingly reveal that the RNase activity is low only in the apical cells, but increases in those beginning to divide (i.e. from the 3rd mm on), whatever base of reference may be used. On the other hand, in the zones of intensively expanding and differentiating cell populations the value of activity per protein-N content of the segment diminishes, but increases per cell and per protein-N unit of it. Consequently, the RNase activity is directly connected partly with the protein-N content of cells and partly with the active cell development (division, expansion). So where the rhythm of development is intensive, e.g. in the apical zones, a high RNase activity can be observed, whatever the reference base may be, and where the rhythm of cell development begins to slow, the enzyme activity shows a high value only, if related to the cell or its protein-N content, because the cells have a relatively large protein-N content. These establishments are also corroborated by the results of HANSON and coworkers [3].

The data presented here prove clearly that between the zones of dividing, expanding cells and those containing entirely differentiated (i.e. developed but vigorously metabolizing) cells, as to enzyme activity a dissimilarity exists. Therefore the RNase enzyme is also suitable for characterizing the developmental stage of the various cell populations and may be considered as a cytological index of root growth.

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DIE ZYTOLOGISCHEN WEISER DES WURZELWACHSTUMS

VI. Änderungen der Ribonuklease-Aktivität in den Zellen von Wurzelsegmenten

Der Verfasser hatte die Aktivität der Ribonuklease (RNase) in 1 mm langen isolierten Segmenten der Maiswurzel untersucht. Die in relativen Einheiten ausgedrückte Aktivität wurde teils auf die einzelnen Segmente, teils auf Protein-N-Einheit bzw. auf eine Zelle bezogen. Die Versuchsergebnisse erbrachten den Beweis, daß es bei der Wertung der Enzymaktivität ratsam ist, sich mehrerer Bezugsgrundlagen (Segment, Zelle, Protein-N) zu bedienen. Die RNase-Aktivität der Zellpopulationen in den verschiedenen Wurzelsegmenten weist in den Zonen des intensiven Entwicklungsstoffwechsels die höchsten Werte auf. In den Segmenten, die mit steigender Entfernung von der Wurzelspitze immer mehr differenzierte Zellen enthalten, nimmt die auf das Segment oder sein Protein-N bezogene RNase-Aktivität ab, steigt aber je Zelle oder je Protein-N-Gehalt dieser an. Die Aktivität des RNase-Enzyms hängt also mit dem Protein-N-Gehalt der Zellen bzw. ihrer Entwicklungsintensität unmittelbar zusammen. Auch ließ es sich feststellen, daß das RNase-Enzym zur Charakterisierung von Zellpopulationen unterschiedlicher Entwicklungsstufe geeignet ist und daher auch als ein zytologischer Weiser des Wurzelwachstums aufgefaßt werden kann.

ЦИТОЛОГИЧЕСКИЕ ПОКАЗАТЕЛИ РОСТА КОРНЕЙ

VI. Изменения рибонуклеазной активности в клетках отдельных сегментов корней

Автор изучал величины рибонуклеазной активности в отдельных миллиметр-овых сегментах корней кукурузы. Выраженную в относительных единицах активность он переводил на отдельные сегменты, отчасти на единицы балкового азота или на клетки. На основе экспериментальных данных при оценке энзиматической активности целесообразно применять несколько основ (сегмент, клетка, протеиновый азот). Рибонуклеазная активность клеточной популяции отдельных сегментов больше всего в зонах с самым интенсивным обменом веществ развития. В более отдаленных от верхушки зонах, содержащих дифференцированные клетки, рибонуклеазная активность в переводе на сегменты или на протеиновый азот сегментов уменьшается, но при переводе на клетки или на протеиновый азот клеток она повышается. Значит, активность рибонуклеазы показывает непосредственную связь с содержанием протеинового азота в клетках или с интенсивностью развития клеток. В то же время активность рибонуклеазы пригодна для характеристики клеточных популяций различной развитости и, следовательно, ее можно рассматривать также как цитологический индикатор роста корней.

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HISTOCHEMICAL STUDIES ON THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN THE CUTANEOUS GUSTATORY EPITHELIA OF FRESH-WATER TELEOSTS

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Synopsis

The distribution of alkaline phosphatase amongst the constituents of the gustatory epithelia of two fishes, *Notopterus notopterus* and *Saccobranchus fossilis* have been studied. The enzymatic activity was seen at the border of the epithelial lining, in the basal layer and in the peripheral zones of the mucous cells and the taste buds. The metabolic significance of the localization of the enzyme at these sites is discussed from the physiological point of view.

Introduction

No doubt, a number of histological studies on the cutaneous gustatory epithelia of various fishes has been done in the past [10, 18, 22, 27, 28, 29, 30, 31] but, to our knowledge, the detailed distribution of enzymes in the constituents of the layers of gustatory epithelia in fishes has not been studied as yet; although a considerable amount of histochemical study has been done by Baradi and Bourne [4, 5, 6] and Bourne [11] on the localization of several dephosphorylating enzymes like acid and alkaline phosphatases, adenosine-triphosphatase, β-glycerophosphatase in the gustatory and olfactory epithelia of mammals including bat, rat, rabbit, monkey and man.

Pevzner [24, 25, 26] has also studied the histochemical localization of a few enzymes including succindehydrase (Succinale NST-reductase) and acetylcholinesterase in the taste buds of certain vertebrates.

In the present study an attempt was made to record in some detail the distribution of alkaline phosphatase among the constituents of the layers of the lips of the fresh-water fishes *Notopterus notopterus* and *Saccobranchus fossilis*.

Material and method

Living specimens of *Notopterus notopterus* and *Saccobranchus fossilis* were obtained from local sources. Small pieces of lips were fixed in either 10% neutral formalin or 85% alcohol or acetone. Paraffin sections were cut at 10 μ. The sections were processed by Gomori's technique for alkaline phosphatase [23]. Suitable controls prepared without substrate in the reaction mixture for alkaline phosphatase were made simultaneously (Fig. 1).

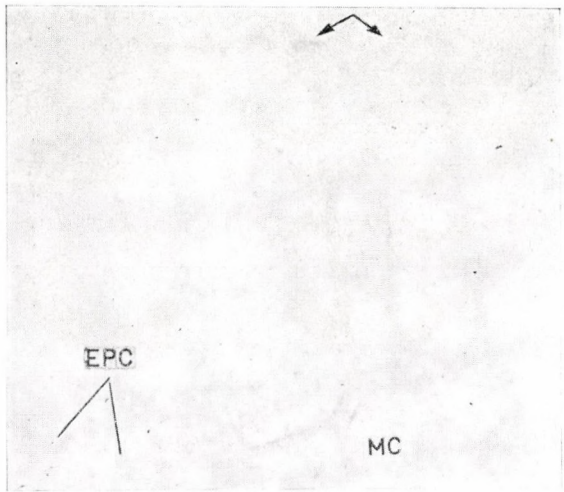


Fig. 1. Control section passing through the ventral surface of the lower lip of *Saccobranchus fossilis*. Note the absence of reaction in the periphery of the epithelium (arrows), epithelial cells (EPC) and mucous cells (MC). ×620

Fig. 2. Section (incubated for longer period) passing through the ventral surface of the lower lip of *Notopterus notopterus*. Note the intense positive reaction in the epidermis. The mucous cells (MC) and dermis (DER) show no enzymatic activity. ×420

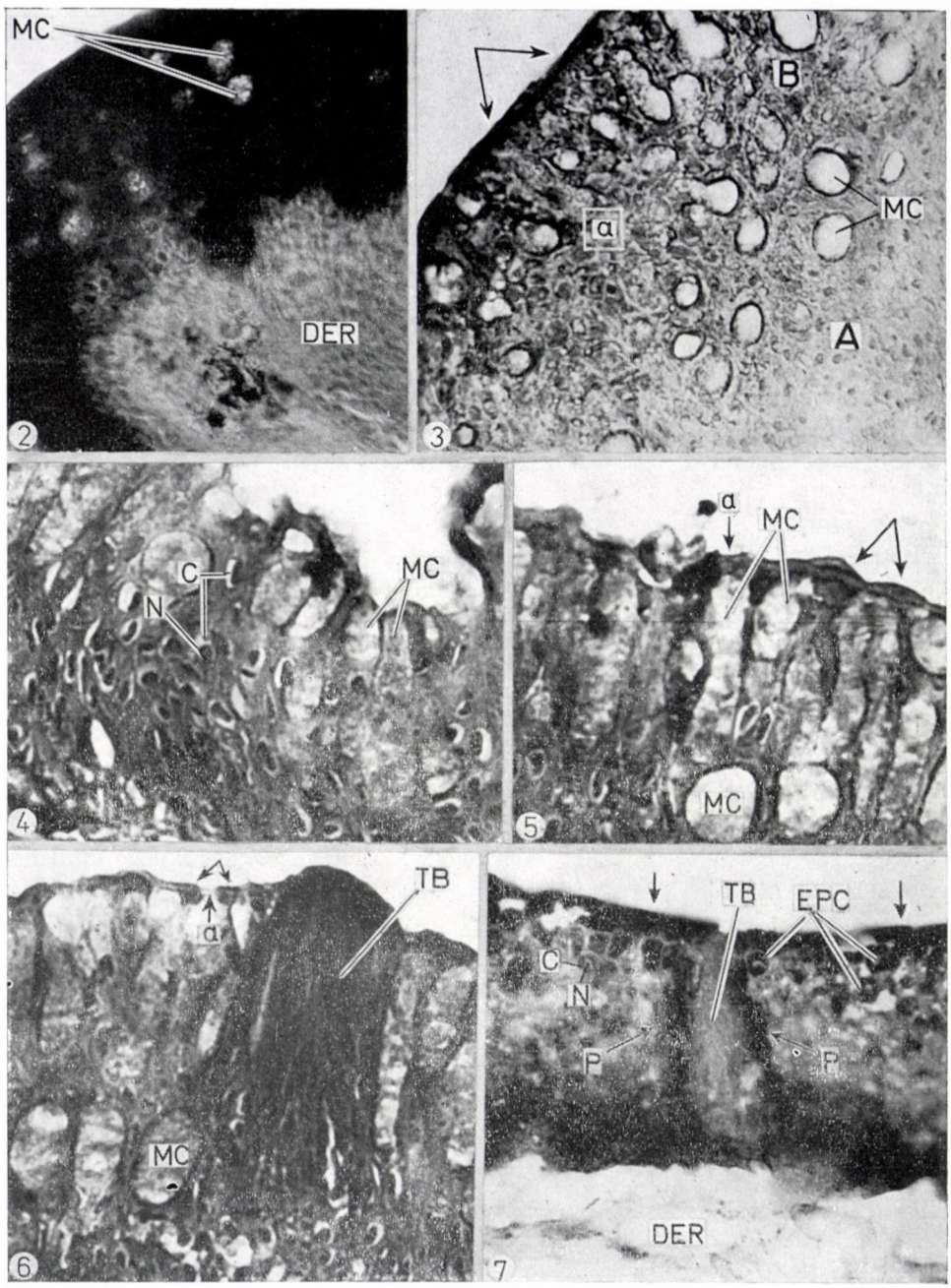
Fig. 3. Section passing through the ventral surface of the lower lip of *Notopterus notopterus*. Note the intense positive reaction at the periphery of the epithelium (arrows). The epithelial cells in middle zones are moderately stained (B zone) or feebly stained in lower portions (A zone). In some epithelial cells note the restriction of the activity to the nuclear and cellular membranes (see within square). Further note the intense positive reaction in a thin rim-like pattern at the peripheries of the mucous cells (MC), the interior portions of these cells are devoid of the reaction. ×430

Fig. 4. A part of the section passing through the dorsal surface of the lower lip of *Saccobranchus fossilis*. Distinguish the positive reaction in the nuclei (N) of the epithelial cells, from the completely negative reaction in the cytoplasmic areas (C). MC = mucous cells. ×600

Fig. 5. Section through the ventral surface of the lower lip of *Saccobranchus fossilis*. Note the intense activity at the border of the epithelium (arrows). The activity in the epidermal portion lying over the mucous cells is of the same intensity (arrows). Distinguish the rounded and elongated mucous cells (MC). ×600

Fig. 6. Section passing through the dorsal surface of the lower lip of *Saccobranchus fossilis*. Note the uniform positive reaction of the alkaline phosphatase at the periphery of the epithelium (arrows) except the areas where the openings of the mucous cells are located (arrow a). Taste bud (TB) gives a strong positive reaction for the enzyme. See the profuse lay-out of the mucous cells (MC). ×600

Fig. 7. Section through the dorsal surface of the lower lip of *Notopterus notopterus*. Distinguish the intense alkaline phosphatase activity at the peripheral (arrows) and basal layers of the epithelium. Note the positive reaction in epithelial cells (EPC) located in the peripheral region. The cytoplasms (C) of these cells are positive while the nuclei are negative (N). The peripheral zones of the taste bud show an intense positive reaction (arrows P). Dermis (DER) is completely negative. ×460



Figs 2—7. Microphotographs representing alkaline phosphatase activity in paraffin sections passing through the lower lips of the fishes *Notopterus notopterus* and *Saccobranchus fossilis;* 10 μ thick (Detailed explanation on p. 270)

Results

Alkaline phosphatase activity in the lips of Notopterus notopterus

Only the epidermis (Figs 2 and 7) has provided a positive reaction. The dermis is completely devoid of the enzymatic activity.

In the epidermis of both the dorsal surface of the lower lip (Fig. 7) as well as the ventral surface of the lower lip (Fig. 3) there is a strong reaction at the outermost layer of the lip constituting the stratified epithelium (arrows; Figs 3 and 7). Staining reaction of equal intensity is provided by the stratum germinativum. The region between these two layers is filled up by the epithelial cells. Amongst these cells the epithelial cells lying in the peripheral portions (EPC; Fig. 7) are intensely positive as compared to the epithelial cells occupying the middle zones which are moderately stained (B zone; Fig. 3) or are feebly stained in the lower portions (A zone; Fig. 3). Further in these cells (EPC; Fig. 7) the nuclear area is devoid of the reaction and the cytoplasm is deeply stained homogeneously. In some cells, particularly in those located in the ventral surface of the lower lip the enzymatic activity is restricted to the nuclear and the cellular membranes (see the cell within square; Fig. 3).

It is also interesting to note that the epithelial cells on the dorsal surface of the lower lip are more positive than the similar cells on the ventral surface of the lower lip (compare Figs 3 and 7).

Mucous cells: These cells are seen scattered among the cells of the epidermis of the ventral surface of the lower lip (MC; Fig. 3); they are absent in the dorsal surface of the lower lip (Fig. 7). In other words, we have found the presence of the mucous cells on the lips of this fish restricted to those surfaces which are in a less close contact with food. Similar observation was published by Al Hussaini and Kholy [2] in *Clarias*. The mucous cells are rounded, oval or flask-shaped (MC; Figs 3 and 8). In haematoxylin-eosine preparations the nuclei of the mucous cells are crescent-shaped and lie at the bases or sides of the cells; they are smaller than the nuclei of the other epidermal cells (Fig. 8). In the enzymatic preparations, however, the nuclei of the mucous cells are not distinguished clearly, presumably due to the intense reaction in the peripheral regions of the cells.

One of the most interesting observations afforded by the present study is that the peripheries of the mucous cells have given positive reaction in the form of thin rim-like pattern whereas the interiors of these cells are completely devoid of reaction (MC; Fig. 3). In certain experiments in which the incubation period was prolonged to many hours, inspite of the long incubation period, no reaction was seen in the central parts of the mucous cells while the intensity of the reaction was increased in the surrounding areas (MC; Fig. 2).

Taste bud: More numerous taste buds have been formed on those surfaces of the lips which are in direct contact with food than elsewhere. This is in

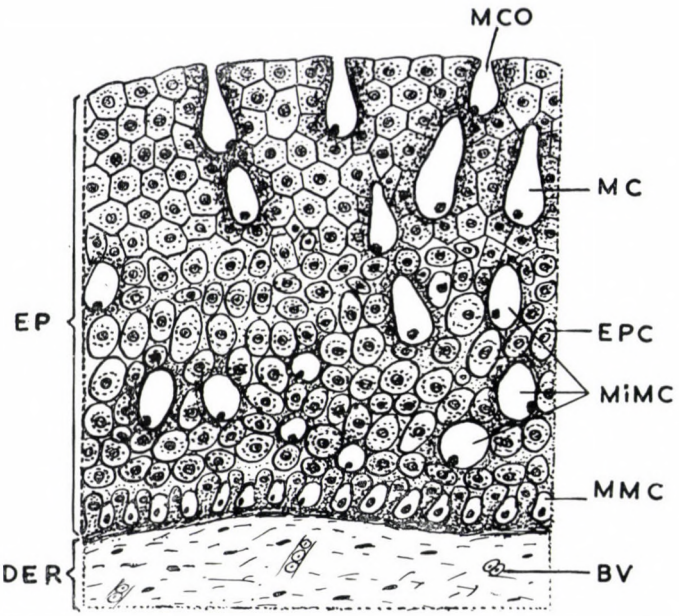


Fig. 8. Diagram of the section passing through the ventral surface of the lower lip of *Notopterus notopterus*. BV = blood vessel; DER = dermis; EP = epidermis; EPC = epidermal cells; MC = mucous cells; MCO = mucous cell opening; MiMC = migrating mucous cells; MMC = mucous mother cell

agreement with the findings of AL HUSSAINI and KHOLY [2]. The peripheral zones of the taste buds have provided intense positive reactions for alkaline phosphatase (arrows P; Fig. 7), like those seen in the superficial layers of stratified epithelium and the layer of stratum germinativum (Fig. 7). The cells within the taste bud (gustatory and supporting cells) seem to be devoid of the enzymatic reaction.

Alkaline phosphatase activity in the lip of Saccobranchus fossilis

Like that of *Notopterus notopterus* the epithelial region of the lip of *Saccobranchus fossilis* gives an intense positive reaction for alkaline phosphatase (Figs 4, 5 and 6). In this fish, too, the outer surface of the lip gives a uniform positive reaction for the enzyme (arrows; Fig. 5) excepting in the areas where the openings of the mucous cells are located (arrow a; Fig. 6). It is interesting to point out here that some of our preparations where the mucous cells have not opened outside the overlying areas (constituted by the epithelial cells) these mucous cells give positive reaction (arrow a; Fig. 5), whereas as soon as the mucous cells acquire their openings (arrow a; Fig. 6) the activity disappears from the overlying areas, presumably due to the loss of the overlying epithelial cells.

In the epidermal cells of *Saccobranchus fossilis* the nuclei are intensely stained for alkaline phosphatase (N; Fig. 4) whereas the cytoplasmic areas (C;

Fig. 4) are completely devoid of the reaction. This is in sharp contrast to *Notopterus notopterus*, in which, reversely, the nuclear areas (N; Fig. 7) are totally devoid of the reaction and the cytoplasmic areas (C; Fig. 7) are intensely positive.

Mucous cells: On the lips of this fish the profuse layout of the mucous cells in the upper half of the epidermal region is seen and this almost com-

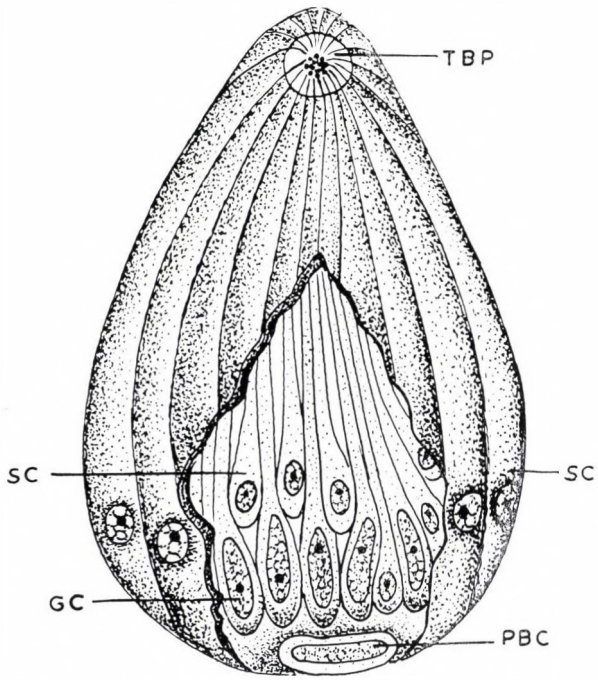


Fig. 9. Diagram of the taste bud showing the arrangement of the cells inside the taste bud. GC = gustatory cells; PBC = peculiar basal cell; SC = supporting or sustentacular cell; TBP = taste bud pore

pletely sandwiches the intervening epidermal cells in this region. Such a profuse arrangement of the mucous cells is not found in *Notopterus notopterus*. The mucous cells on the lips of *Saccobranchus fossilis* are somewhat larger than those in *Notopterus notopterus* but in shape the mucous cells of the two species are similar. In *Saccobranchus* the cells in the middle of the epithelium are rounded (MC; Fig. 5) or oval in shape (MC; Fig. 6), while the cells located towards the periphery of the epithelium are somewhat elongated (MC; Figs 4 and 5).

In this fish, too, the periphery of the mucous cells has shown a strong positive reaction as a thin rim-like positive zone, whereas the interiors of the cells are devoid of the reaction. Unlike the condition seen in *Notopterus notopterus*, in *Saccobranchus* mucous cells are present on both surfaces of the lip.

Taste bud: The taste buds on the lips of *Saccobranchus* are much more in number than those on the lips of *Notopterus* and are distributed almost equally on all the surfaces of the lips. Fig. 9 depicts a three-dimensional aspect of a taste bud. From this it is apparent that the supporting or sustentacular cells (SC; Fig. 9) constitute the outer covering of the cup-like taste bud. In the interior regions are localized the gustatory cells (GC; Fig. 9). Our histochemical preparations point out that the peripheral localization of the enzyme, seen in the section, may indicate a location of the enzyme in the region covering the taste bud, in other words, in the regions occupied by the supporting cells. In Fig. 7 the interior of the taste bud, i.e., the regions where the gustatory cells are located have not provided a reaction. In Fig. 5 the reaction is so intense that it is difficult to distinguish whether the gustatory cells are positive or not. Nevertheless a comparison between Fig. 9 and Figs 6 and 7 leave little doubt about the enzyme activity of the supporting cells.

Discussion

In both of the fishes examined the peripheries of the epithelia of the dorsal and ventral surfaces of the lower lips are equipped with alkaline phosphatase activity, in this respect being different from the epithelia of man, rat, bat, rabbit and monkey [5, 12, 16, 17, 32]. The presence of alkaline phosphatase at the peripheral portion of the epithelium, in contrast to its absence in mammalian epithelia, can be linked possibly to two factors, firstly, in the fishes the outer covering is not keratinized, it consists of living cells; secondly, these cells are in constant contact with water. The latter fact makes it imperative that substances in the surrounding medium must diffuse through this zone. The location of the alkaline phosphatase at these sites acquires special significance in this context. It is quite possible that the enzyme may play an important metabolic role in facilitating the transport of substances across the membranes. The role of the alkaline phosphatase in the absorption of sugar from the intestine and in the reabsorption of glucose in the kidney tubules has been emphasized by CANTAROW and SCHEPARTZ [14]. HASHIMOTO and OGAWA [17] are also of the opinion that the alkaline phosphatase intervenes in the adjustment of the permeability, and in the exchange of substances through the cell membrane. MÖLBERT and co-workers [21] also support the above contention on the basis of their observation by electron microscopy: they detected alkaline phosphatase in the cell membrane of kidney cells. In other tissues the role of alkaline phosphatase in the permeability processes across the cellular membranes has also been noted [13]. TEWARI and BOURNE [33, 34] have linked the role of alkaline phosphatase with the permeability processes at the neuronal surfaces.

It is well known that new cells are made up in the basal layer and in this layer extensive *de novo* protein synthesis takes place [9]. A number of studies

have pointed out that alkaline phosphatase is associated with the formation of fibrous proteins [13]. HASHIMOTO and OGAWA [17] also point out that alkaline phosphatase plays an important part in transphosphorylation in the young metabolically active parts of the tissues. In view of these observations the presence of alkaline phosphatase in the basal layers of the skin, as shown in the present studies, points out the role of the enzyme in active protein synthesis associated with the proliferation of the cells in this zone.

However, the differentiated cells, as they migrate towards the upper region, seem to differ in the two fishes, as far as their enzymatic equipment is concerned. In *Notopterus notopterus* the nuclei are devoid of the enzymatic activity, whereas the cytoplasm is generally intensely positive; very rarely cells have been seen where the alkaline phosphatase is absent from the cytoplasm and the enzyme is restricted at the nuclear and cellular membranes. On the other hand, in *Saccobranchus fossilis* the differentiated epidermal cells present a pattern just opposite the ones seen in *Notopterus notopterus*, viz., the nuclear areas are intensely stained and the cytoplasmic areas are totally negative. It is difficult to explain these differences at present. It may be supposed that in our sections of the *Saccobranchus* we have caught the stage where at the particular moments the alkaline phosphatase in the nuclei was playing its role in the nucleic acid synthesis [35, 36].

Regarding the significance of the presence of alkaline phosphatase in the cytoplasm of the epithelial cells of the upper zone, experimental studies with labelled compounds have shown that synthesis of RNA can occur, besides the stratum germinativum, in the cells of the other layers of the epidermis [9]. Since it has been demonstrated that alkaline phosphatase is concerned with the nucleic acid, protein and phospholipid synthesis in a variety of cells [19, 35, 36, 37], our results point out that the alkaline phosphatase may be associated with such phenomena in these cells.

The mucous cells, which form the conspicuous and abundant slimes of fish, have been recognized as the epithelial cells which migrate from the basal layer (see Fig. 8) and further it has been suggested that as they migrate upwards the nuclei get pushed towards the bases of the cells and simultaneously the interior gets filled up with the mucus [3]. AL HUSSAINI [1] also supports such a contention and remarks that in the mucous cells mucous secretion commences very early, during the course of their differentiation from the basal layer and the amount of secretion gradually increases as the cells approach the surface. Our preparations show heterogeneity in the mucous cells from the point of view of the enzymatic reaction. The central parts of the mucous cells are completely devoid of the reaction and the intense enzymatic activity is seen restricted to the peripheries of the mucous cells. These sites represent the areas where nuclei and mucus-free cytoplasm are present.

It has been postulated that mucin-synthesis takes place in the cytoplas-

mic area above the nucleus [20] and, therefore, it seems reasonable to suppose that the alkaline phosphatase at these sites may have something to do with the process of secretion. Furthermore, the possibility of drawing the metabolites from the surrounding cells by the peripheral zones of the mucous cells may also exist. If this process really takes place, then the localization of the alkaline phosphatase may mean that the enzyme is playing its role in the facilitation of the substances across the membranes of the mucous cells. It may be added here that the peripheral location of the enzyme continues to be seen in those mucous cells which have become elongated and open outside for the ejection of the mucous material.

In the present study the alkaline phosphatase has been seen in the peripheral parts of the taste buds. As already pointed out these are the regions which are occupied by the supporting cells. The significance of the localization of alkaline phosphatase at these sites can be speculated in two ways. Firstly, the enzyme may facilitate the passage of metabolic substances from the surrounding zones to the interior of the taste buds through the wall. Secondly, it has been shown in a number of experimental studies with labelled compounds that the cell division occurs in the periphery of the taste buds and differentiated cells migrate into the central area of the taste bud [7, 8, 15]. Since there is augmentation of protein synthesis at the time of division of cells and furthermore, as the role of alkaline phosphatase has been linked with protein synthesis [37] the presence of intense activity in the peripheral parts of the taste buds may be hinting the role of the enzyme in such processes.

BARADI and BOURNE [4] have studied the localization of several dephosphorylating enzymes in the taste buds of the rabbit and other mammals (monkey, bat). They concluded that the epithelium which lines the gutters of lingual papillae appears to contain several such enzymes (acid and alkaline phosphatase, adenosine triphosphatase, β-glycerophosphatase). Some of these are located at the base of the bud, others at the apex. The cells themselves are poorer in enzyme than the surrounding epidermal cells. In this respect our results disagree. We have seen intense localization of the enzyme in the zone occupied by the supporting cells.

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HISTOLOGISCHE UNTERSUCHUNG DER ALKALISCHEN-PHOSPHATASEN-VERTEILUNG IM GESCHMACKSEPITHEL DER HAUT VON SÜSSWASSERFISCHEN DER UNTERKLASSE TELEOSTEI

Verfassor untersuchten die Verteilung der alkalischen Phosphatase in den Bestandteilen des Geschmacksepithels zweier Fischarten, der *Notopterus notopterus* und *Saccobranchus fossilis.* Eine Enzymaktivität ließ sich an der Grenze der Epithelauskleidung, in der Basalschicht und in den peripheren Zonen der Mukosazellen, sowie in den Geschmacksknospen nachweisen. Die Bedeutung der obigen Lokalisation der alkalischen Phosphatase für den Stoffwechsel wird aus physiologischer Sicht erörtert.

ГИСТОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ РАСПРЕДЕЛЕНИЯ ЩЕЛОЧНОЙ ФОСФАТАЗЫ В ВКУСОВОМ ЭПИТЕЛИИ КОЖИ ПРЕСНОВОДНЫХ РЫБ ПОДКЛАССА TELEOSTEI

Авторы изучали распределение щелочной фосфатазы в составных частьях вкусового эпителия двух видов рыб *Notopterus notopterus* и *Saccobranchus fossilis.* Ферментативная активность была выявлена на границе эпителиальной подкладки, в базальном слое и в периферических зонах слизистых клеток, а также во вкусовых почках. Обсуждается значение указанной локализации щелочной фосфатазы для обмена веществ с точки зрения физиологии.

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FINE STRUCTURE OF THE PHARYNX AND ENDOSTYLE OF AN ASCIDIAN (CIONA INTESTINALIS)

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Synopsis

Structure of the cells of the endostyle and the branchial bars in the pharyngeal wall of *Ciona intestinalis* have been investigated by electron microscopy. In the endostyle zone I can be divided into axial and lateral cells, both cell types re presenting a mucus-producing ciliated epithelium. Zones II and IV consist of glandular cells of the same type which produce a granular secretion of unknown nature. Zone VI is formed by glandular cells the apical pole of which accumulates great masses of a special granule. Zones III, V. and VIII are built up of ciliated cells; those in zones III and V bear also regularly arranged microvilli. In cells of the squamous epithelium of zone VII the rough-surfaced endoplasmic reticulum contains a fine floccular material and, sometimes, some osmiophilic granules. In the cytoplasm of the same cells a great number of lipofuscin-like inclusions can be found. In the epithelial bars of the pharynx ciliated and glandular cells of various secretion phases were observed. Between zones VII and VIII there are several cells resembling the glandular cells in the epithelial bars of the pharynx.

Introduction

The endostyle represents a special organ in the pharynx of Ascidia, Cephalochordata, Cyclostomata and certain Prosobranchiata. In *Ciona intestinalis* it forms a trough-shaped structure in the ventral wall of the pharynx which extends from the fore-part of the pharynx to the esophagus. Barrington [1] distinguished eight symmetrical longitudinal layers (cell-zones) in the endostyle. These separate zones consist of glandular, ciliated and squamous epithelial cells (Fig. 1).

The endostyle of the different ascidian species has been investigated by numerous authors [5, 6, 10, 14, 21, 22, 23], still there exist many undecided problems concerning its morphology and function. Zone I is generally considered a mucus-producing zone [1, 11]. It has been suggested [16] that other zones may also participate in the mucus production. In the opinion of several investigators, the different glandular zones secrete different products [1, 12, 16, 22], but the histochemical results regarding these substances are inconsistent. Secretion transport is attributed to the ciliated zones.

Some comparative anatomists have considered the endostyle homologous to the thyroid gland of the vertebrates [25], but according to Barrington and co-workers [1, 2, 3, 4], zone VII is the only zone of the endostyle which is

capable of incorporating iodine and, therefore, only this zone is regarded as analogous with the thyroid gland.

The complicated cellular composition of the endostyle, the scarcity of the data on its function and the contradictory data of the light-microscopic investigations, prompted us to perform an electron-microscopic study of this organ. LEVI and PORTE [11] have investigated the fine structure of the endostyle in another tunicata *(Microcosmus claudicans)*. Their study, together with the present work, may serve as a morphological basis for further experimental investigation of this organ.

Material and method

Specimens of *Ciona intestinalis* were collected from the Golf of Naples and the Adriatic Sea in September. They were transported to our laboratory in cooled sea water of 10—15°C, where, 10 hours after the collection, the pharynx region containing the endostyle was fixed in a solution prepared as follows: to 8 g of paraformaldehyde dissolved in 94 ml warmed sea water of double concentration, 20 drops of *N* NaOH, 40 ml of 0.2 *M* cacodylate buffer, 6 ml of 50% glutaraldehyde diluted with 6 ml sea water of double concentration and distilled water ad 200 ml were added. The pH of the solution was at 7.6—7.7. It should be noted that we have tested numerous other fixatives, but the structure of the endostyle was not satisfactorily preserved in these. On the other hand, the above fixative is not apt at all for preservation of the nervous system and the neural gland of the animal.

From the fixed material small pieces were cut out in the transversal plane and postfixed for 1 hour in 1% osmium tetroxide dissolved in phosphate buffer [13]. The material was dehydrated in alcohol, embedded in Araldit and examined in a JEM 6C electron microscope.

Results

All the eight zones described by BARRINGTON [1] were examined. The localization of these zones, their shape and some characteristics of their structure can be observed also with light microscope in the sections of 1—2 μ thickness prepared in the same way as for electron microscopy and stained with toluidine blue (Fig. 1).

Zone I. This zone forms the base of the endostyle. In accordance with the light-microscopic studies of SOKOLSKA [22] and the electron-microscopic ones of LEVI and co-workers [11], two cell types were distinguished in it.

Axial cells: ciliated cells of a slender, cylindrical shape; the basis of the cells is attached to the basal membrane of the endostyle. Their apical surface bears long cilia, each surrounded by numerous short microvilli. The cytoplasm contains a great number of mucus droplets accumulating at the apical pole (Fig. 2). In the basal region long tubuli of the rough-surfaced endoplasmic reticulum and small mitochondria with cristae are situated. In some vesicles of the Golgi apparatus a substance of variable density — sometimes as dense as the mucus droplets — can be observed.

Lateral cells: these are located above the axial cells on the surface of the cells of zone II (Fig. 1, Ib) and differ from the axial cells in their less uniform shape. The cells located nearer the base of the endostyle have a cuboidal shape,

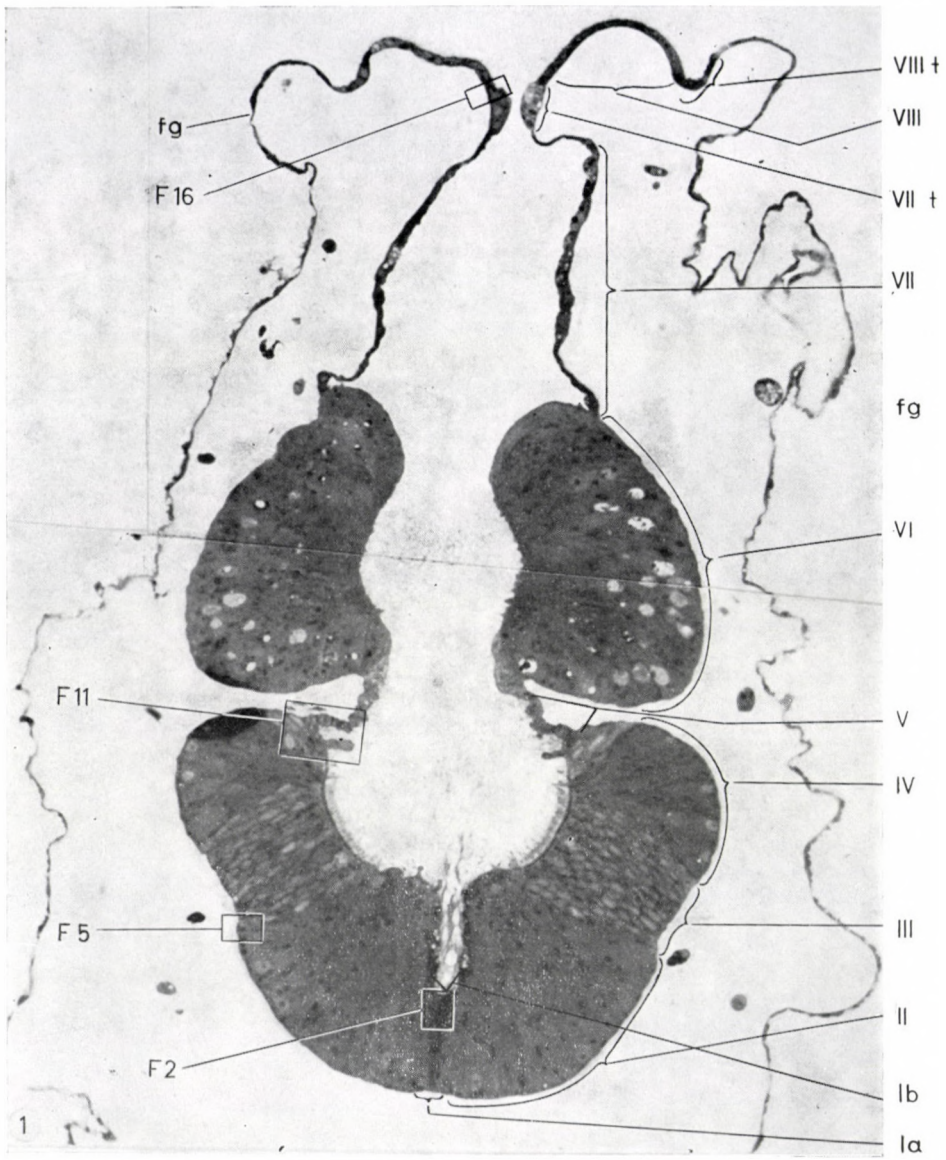
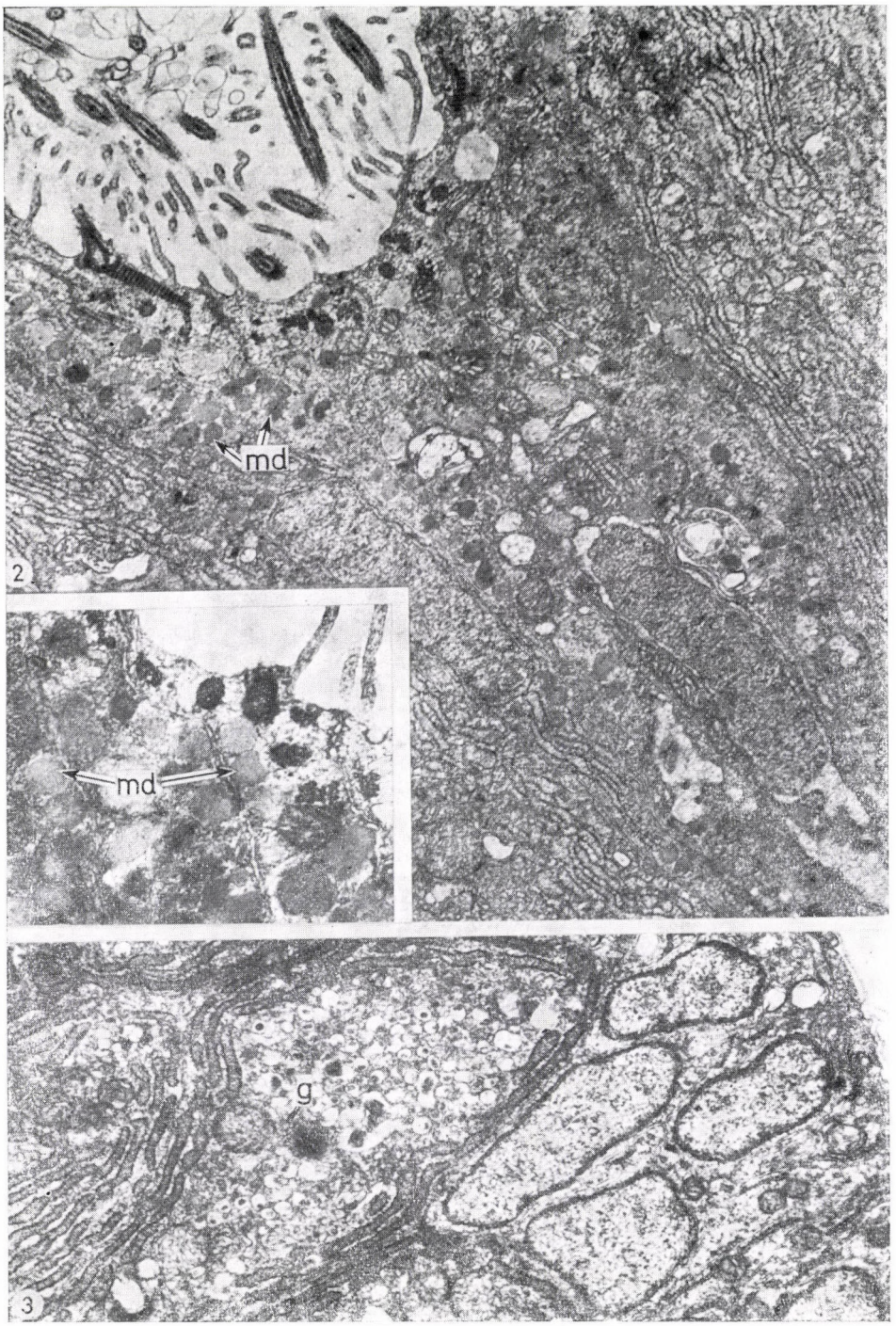


Fig. 1. Photomicrograph of the endostyle and a portion of the gill-chamber's wall (fg). The different zones are marked, at the right side, with corresponding numerals and letters. At the left side the numbers of the electron micrographs, photographed from sections of the same block are marked (Figs 2, 6, 14, 21). ×460

a single cilium and a few microvilli protruding from their surface into the lumen. The nuclei are spherical, the cytoplasm contains numerous mucus droplets and many tubuli of the rough-surfaced endoplasmic reticulum, variable in diameter.

Moving from the base towards the edge of zone I: more flattened lateral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cells with less differentiated cytoplasms are seen until the mucus droplets and the tubuli of the endoplasmic reticulum disappear. The cells at the edge of zone I are rich in free ribosomes, have no cilia and only few microvilli.

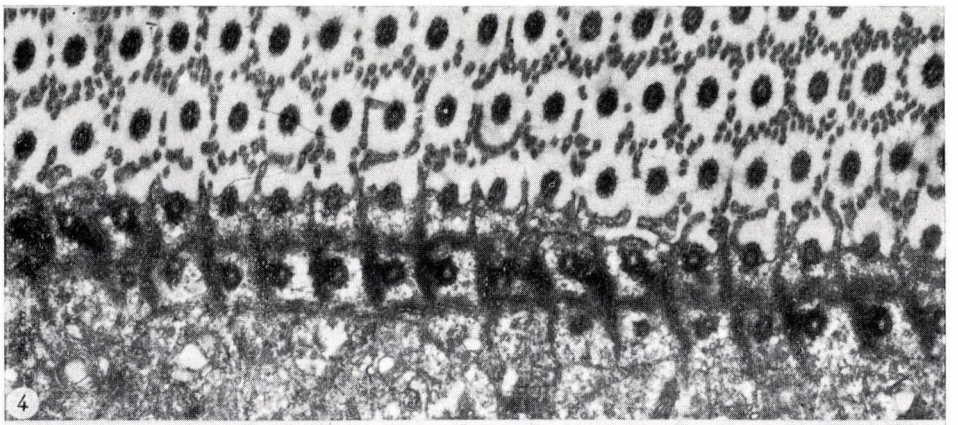
Zone II (Fig. 1, II). It contains elongated cells tapering in the apical direction, bearing long microvilli, some of them also cilia, at their surface. At the base of the microvilli complex vesicles can often be observed at the cell membrane (Fig. 20). As the chief component, the cytoplasm contains a well-developed ergastoplasm with approximately parallel membranes extending from the base to almost the apical pole. Between membranes of the ergastoplasm mitochondria of tubular structure are scattered and, in smaller and larger groups, secretion granules can be found. The secretion granules at the apical poles of the cells are spherical, 200 mμ in diameter, have a smooth-surfaced limiting membrane and contain a dense osmiophilic substance; between the osmiophilic substance and the limiting membrane there is a clear zone. The granules in the basal half of the cells on the other hand are mostly oval, 3—4 times as large as the apical ones, since the clear zone surrounding the osmiophilic substance 200 mμ in diameter is extremely wide. The osmiophilic content within the granules is usually eccentrical and often shows a loose structure (Fig. 3). In the cytoplasm a compact body of unknown nature, 600 mμ in diameter, may occur. It is bordered by a membrane.

Zone III. This zone is very rich in nuclei (Fig. 1, III). Its cells are slender, ciliated and poor in organelles. The apical cell-surface is quadratic the side of the squares being 700—800 mμ. In the centre it bears a single cilium and, at the edges, 14—16 microvilli. The microvilli encircle the cilia like a wreath and run parallel with them for a long distance (Figs 4, 5). At the apical pole the cells are attached to one another by junctioning structures of zonula adhaerens type equipped with tonofilaments. The ciliary rootlets are oriented in the same direction. In the cytoplasm elongated mitochondria with cristae, some scattered tubuli of the rough-surfaced endoplasmic reticulum, free ribosomes and a little Golgi apparatus are observable. The Golgi region contains, relatively often, some osmiophilic bodies maximally 350 mμ in diameter (Fig. 8).

Zone IV (Fig. 1, IV). The cells in this zone have a cytoplasmic structure and secretion granules identical with those in cells of zone II. In zone IV, however, the apical pole of the cells contains more secretion granules. At the surface of this zone discharged granules and complex vesicles are more often found, otherwise the cell surface resembles that in zone III (Figs 6—10).

Fig. 2. Zone I. Axial cells from the region F2 of Fig. 1. Above the nucleus and in the inset mucus droplets (md) are visible. ×10,200; inset ×22,000

Fig. 3. Adjoining of basal halves of zones II and III at the region F5 of Fig. 1. Note the characteristic secretion granules (g) on the basal half of zone II. Zone III, rich in nuclei, is visible at the right side of the Fig. ×13,200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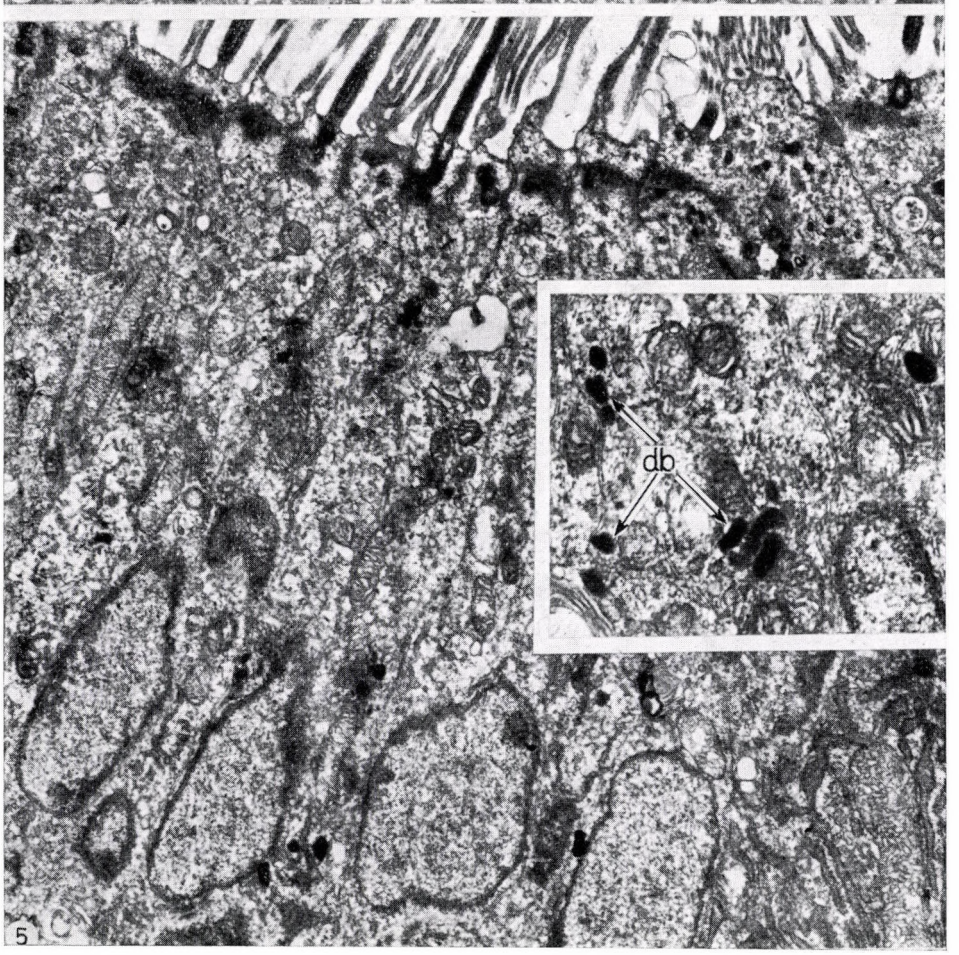
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Zone V is built up of small, cylindrical ciliated cells. Its endings attached to zones IV and VI are widened, the nuclei being arranged at these sites in several rows. The typical cylindrical cells are located, in the middle region of the zone, in a single layer (Fig. 1, V). The relative amount of the cytoplasm is little. It is poor in organelles and resembles that of the cells in zone III. Compact osmiophilic bodies are to be found also in this zone. At the cell-surface the microvilli are short, less numerous and show no regular arrangement around the cilia. On one of the cells only one cilium was found (Fig. 11).

Zone VI is the most developed glandular zone (Fig. 1, VI) formed by cylindrical glandular cells containing abundant ergastoplasm and special secretion granules. The surface of the cells bears short microvilli in an irregular arrangement. Some of the cells are ciliated. The apical pole of the cytoplasm is filled with spherical secretion granules of variable density and a diameter of 600 mμ or less. The larger granules, denser than the smaller ones, have the limiting membranes closely attached to their surface. The osmiophilic material of the smaller granules is separated from their limiting membrane by a clear zone (Fig. 13). The nuclei, each containing a large nucleolus, are located near the base. The mitochondria are large and have a tubular structure as in the other glandular zones.

In the basal half of cells of zone VI the light microscope reveals spherical bodies several μ in diameter (Fig. 1) which show a yellowish-brown colour in native preparation. In electron micrographs granules 200—400 Å in diameter can be observed within these bodies. Some granules are so close to one another that it is almost impossible to mark off them from each other; others are situated at smaller or larger distances from each other. Glutaraldehyde fixation [15], may produce artefacts as vacuoles and membrane-configurations (Fig. 12).

Zone VII. is built up of squamous epithelial cells of 1.5—2 μ thickness which are attached to one another by deep interdigitations. Their cell surface shows no signs of any specialization apart from one or two microvillus-rudiments. The cytoplasm contains few organelles. The rounded mitochondria are scattered and contain cristae as an inner structure. The cytoplasm is rich in free ribosomes. Scattered tubuli and cisternae of the rough-surfaced endoplasmic reticulum enclose a fine floccular material condensed, here and there, into bodies similar to osmiophilic granules (Fig. 14). Also some vacuoles of the Golgi apparatus contain a dense osmiophilic substance. In the cytoplasm, moreover, numerous dense bodies covered with a membrane and having a size of 1 μ or less

Fig. 4. Zone III. Oblique section from the apical poles of the cells. Cilia and microvilli in regular arrangement. ×11,200

Fig. 5. Zone III. Longitudinal section showing the cytoplasm above nucleus and the cell surface. At the upper right corner the apical portion of a cell of zone II is visible. ×9,000. Inset: "dense bodies" (db) in the perinuclear cytoplasm of a cell in zone III. ×21,500

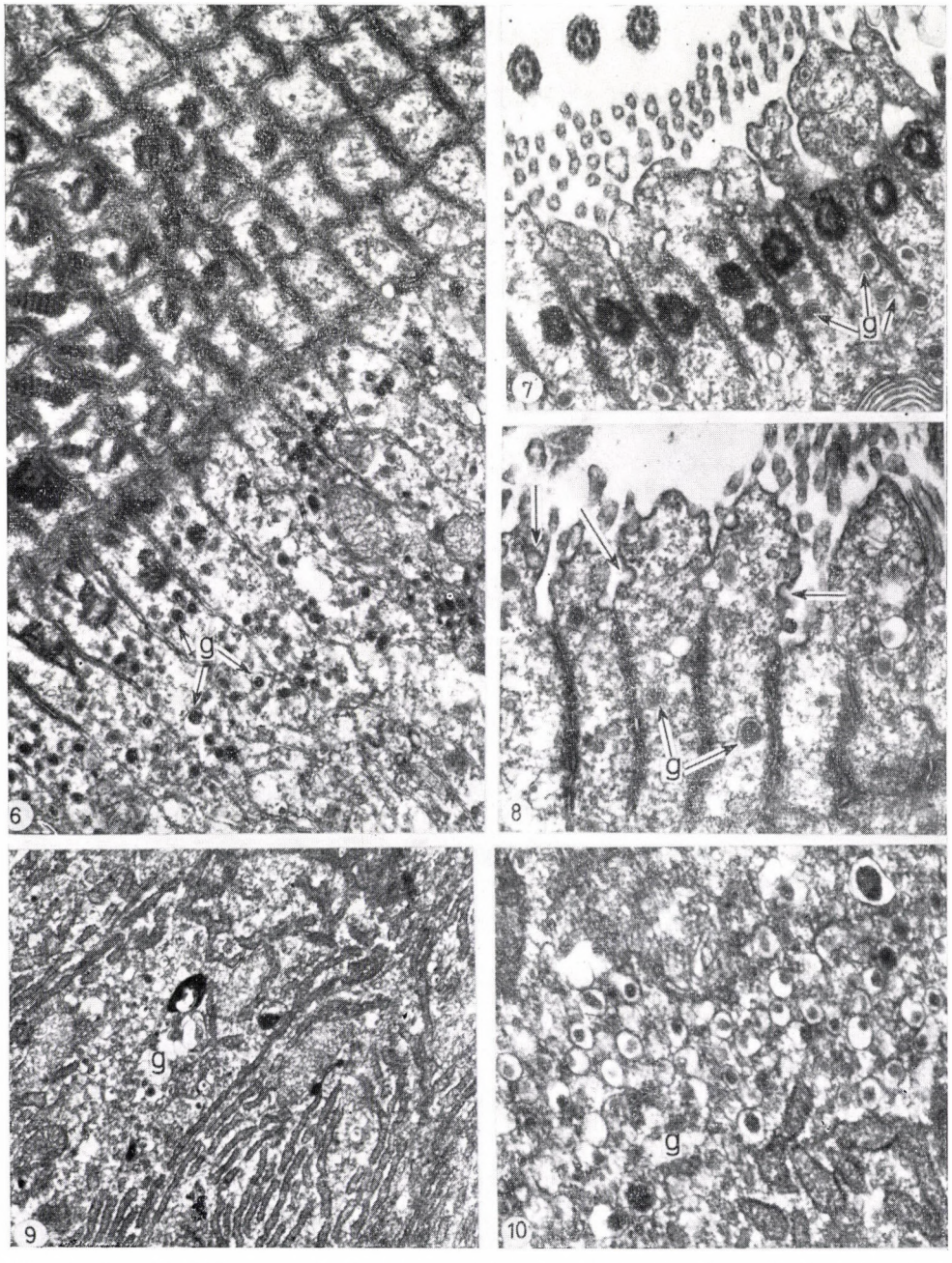

Fig. 6. Zone IV. Oblique section from the apical halves of the cells. Note the ciliary rootlets, the secretion granules (g) and the large tubular mitochondria. ×15,600

Figs 7 and 8. Zone IV. Apical pole of cells with secretion granules (g). Note the specialized cell surface. The arrow indicates complex vesicles. ×21,000

Figs 9 and *10.* Zone IV. Ergastoplasm and secretion granules (g) in the basal half of the cells. Fig. 9: ×8,700, Fig. 10: ×22,300

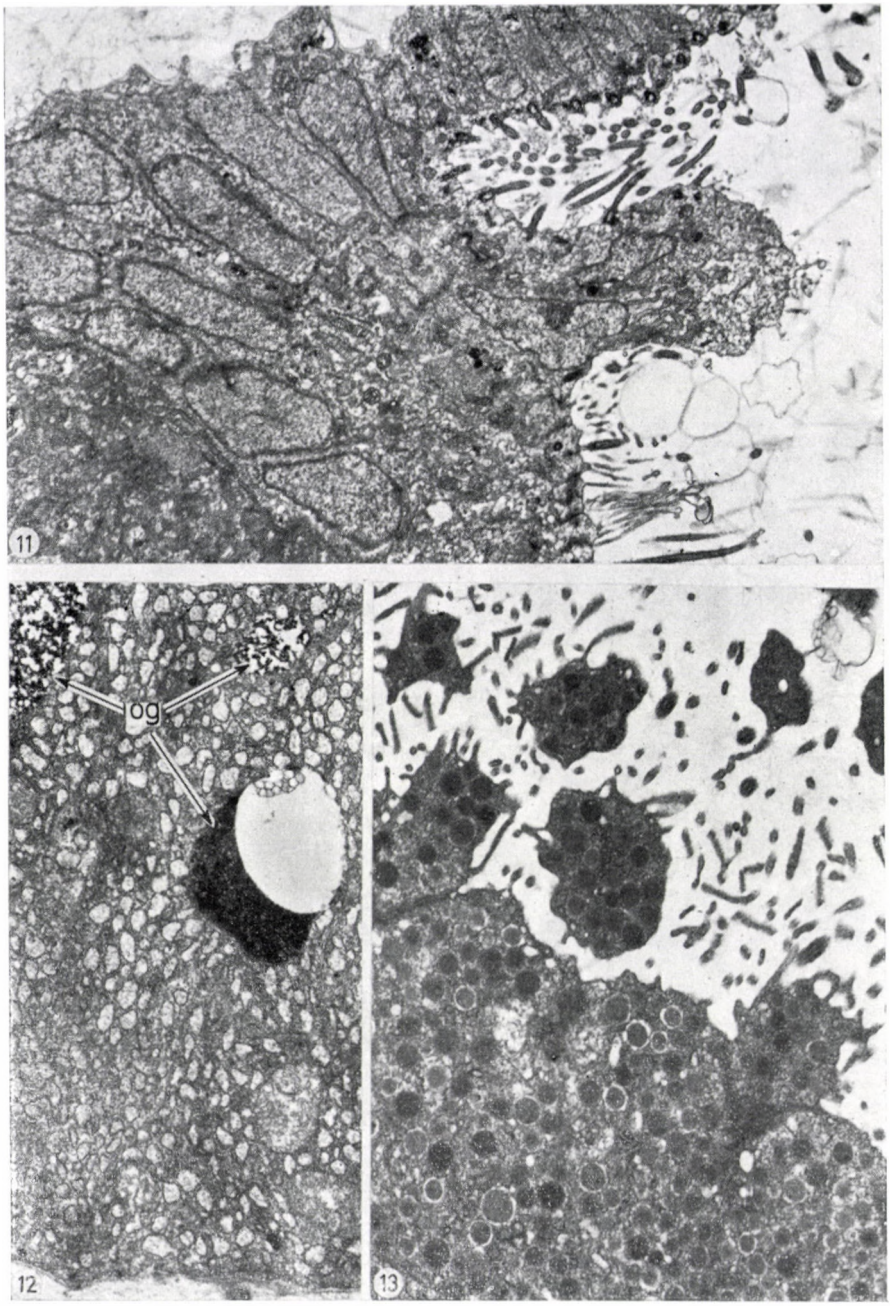

Fig. 11. Zone V. Region F11 of Fig. 1. Approximately cylindrical ciliated cells different in size, bearing microvilli. ×4,000

Fig. 12. Zone VI. The basal part of the cells. The basal membrane, mitochondria, ergastoplasm and osmiophilic granules (og) condensed in variable degree are visible. ×8,640

Fig. 13. Zone VI. Apical pole of the cells is filled with large secretion granules. ×10,000

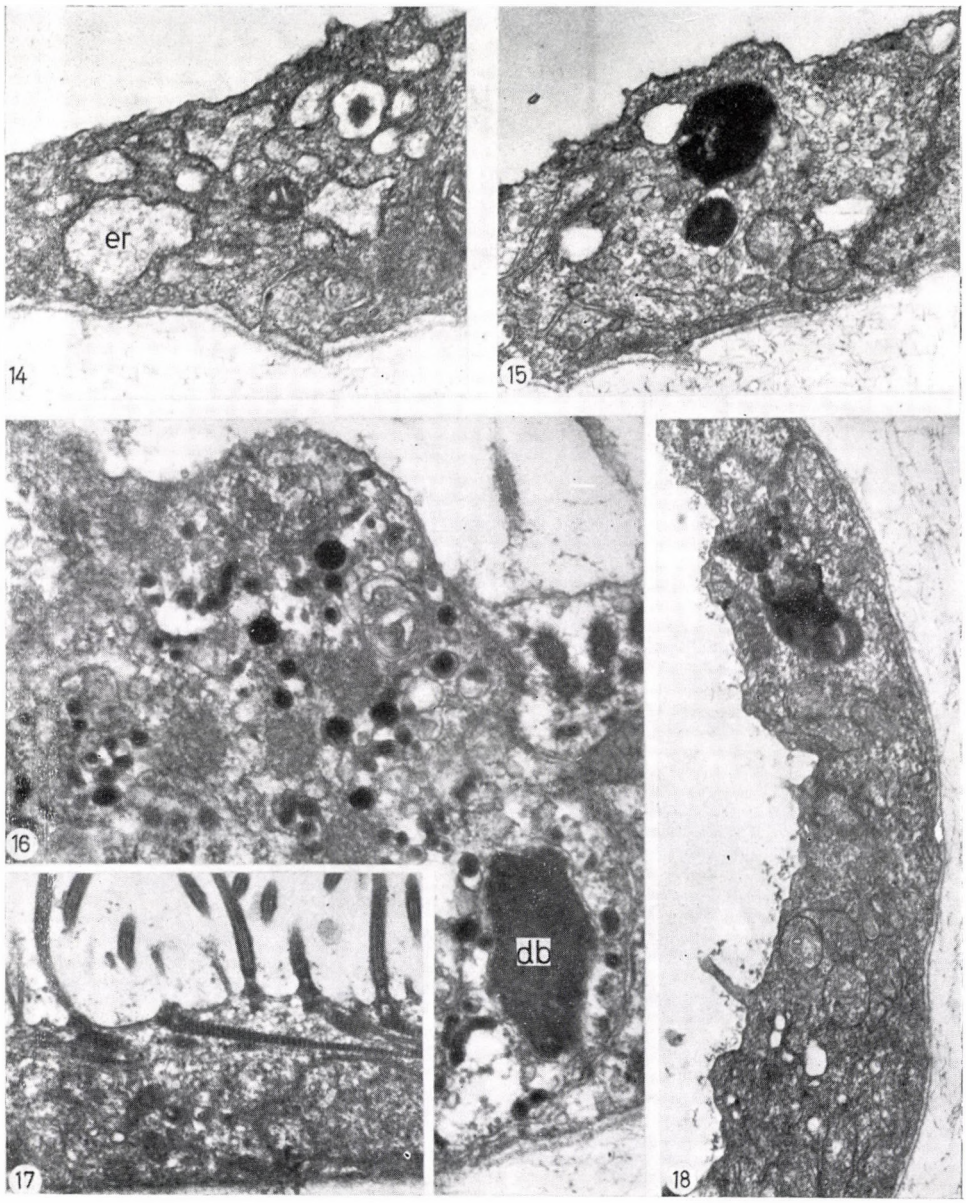

Fig. 14. Zone VII. In broadened cisternae of the rough-surfaced endoplasmic reticulum a fine granular substance can be seen (er). In a profile a dense osmiophilic substance is located. ×24,500

Fig. 15. Zone VII. Dense bodies of variable size, covered with a membrane. ×19,000

Fig. 16. Zones VII and VIII from region F16 of Fig. 1. On the left, one of the terminal cells of zone VII is shown with numerous secretion granules and a large "dense body" (db). ×25,400

Fig. 17. Zone VIII. Typical ciliated cells. ×10,250

Fig. 18. First epithelial cell of the gill-chamber beyond zone VIII, having the same structure as cells of the zone VII. ×12,700

in diameter are to be found (Fig. 15). These bodies resemble the lipofuscin granules or the residual bodies of other animals (Figs 19 and 20).

Between zones VII and VIII a special cell-type was found which has been termed terminal cells of zone VII (Fig. 1, VII t). In cross section of the endostyle 2 or 3 of these cells can be found beside each other. Morphologically they are cuboidal or low cylindrical epithelial cells of undifferentiated surface, with rounded nuclei at the basal region. Their cytoplasm contains a great number of secretion granules 250 mμ in diameter and bordered with a membrane. Among them several larger bodies of variable shape and of an osmiophilic dense substance are observable (Fig. 16).

Zone VIII (Fig. 1, VIII) is formed by flattened cells bearing numerous cilia. The long ciliary rootlets are directed towards the base of the endostyle. Among the cilia some microvilli are dispersedly protruding from the surface (Fig. 17).

Other cell types of the pharynx. Beyond zone VIII the wall of the gill-chamber is coated, in a width of 20—30 μ with cells characterizing zone VII. These cells have been termed terminal cells of zone VIII (Fig. 1, VIII t and Fig. 18).

The gill-chamber represents an organ of enormous size when compared with the body of the animal. Its wall is coated, beyond the terminal cells of zone VIII, with flattened thin cells (Fig. 1, fg) which form, here and there, thickened epithelial bundles discernible to the naked eye. The epithelial bundles extend in longitudinal direction from the mouth to the esophagus; light microscope shows ciliated cells in them.

The electron microscope makes ciliated and glandular cell groups visible in the epithelial bundles. The former are identical with those in zone VIII of the endostyle, the latter resemble the terminal cells of zone VII. In epithelial bundles of the gill-chamber, however, the glandular cells are in conspicuously different phases of secretion and, in accordance with this, they contain secretion granules and ergastoplasm in different quantities. In ergastoplasmic cisternae of the regranulating cells a fine floccular substance can be found. In the cytoplasm of the same cells also some dense osmiophilic bodies larger than the secretion granules are contained. The apical pole of the strongly granulated cells is totally filled up with dense secretion granules 250—300 mμ in diameter and spherical in shape. The glandular cells are smooth-surfaced and some of them bear one or two cilia (Figs 19 and 20).

Discussion

Cell types in the different zones of the endostyle

The so-called glandular zones. Zone I. In conformity with the observations of Sokolska [22] on different ascidian species and with those of Levi and Porte [11] on *Microcosmus claudicans*, axial and lateral cells were found in this zone.


gc
gc
cc
19
db
20

On the basis of histochemical investigations, BARRINGTON [1] attributes mucus production to cells of zone I in *Ciona intestinalis*. Mucus-producing cells have been observed also in other zones of the endostyle of other animal species by histochemical methods [11, 16]. We have found mucus droplets only in axial cells and in lateral cells situated near the base of zone I, thus, our results support the view of BARRINGTON [1].

At the upper border of the lateral region of zone I, the cells are undifferentiated regarding both the cytoplasm-structure and the cell-surface. We suppose that the differentiated cells situated more deeply in the zone are replaced by these cells.

In zones II and IV the cytoplasm-structure and the secretion granules of the cells have proven as identical. It is possible, therefore, that in these two zones a secretion of the same nature is produced. The chemical composition and physiological role of the secretion are unknown. In both zones the cell surface is differentiated; it bears, especially in zone IV, besides cilia, microvilli in an extremely regular arrangement. This resembles the superficial differentiation of zones II and IV in *Microcosmus claudicans* [11]. Presumably, the microvilli have a role in the resorption of some substances rather than in the secretion processes [20].

It is remarkable, from morphological point of view, that in the apical and basal halves of zones II and IV the size and structure of the secretion granules are different. These differences may arise from that in basal half of the cells the granules are immature and their structure loosened. In addition, they may undergo some swelling during preparation.

Zone VI is the widest glandular zone in the endostyle and its cells are the richest in secretion granules. The size and structure of the granules differentiate the product of this zone from that of the other glandular zones; its more detailed analysis is still waiting to be performed. Also the granules in the basal half of the zone are of unknown composition and function. These cannot be kept for precursors of the mature apical granules and unlike the granules in zone VI of *Microcosmus claudicans* [11], they are not identical either with the lipofuscin granules of higher animals.

The so-called ciliated zones. Zones III, V and VIII belong to this group. The cytoplasmic structure and cell surface of these zones point first to a mechanical and, secondly, to a resorbing function. As it was seen, cells of zone III are ciliated and have microvilli arranged in an extremely regular way (Figs 4 and

Fig. 19. Portion from the epithelial bar of the gill-chamber showing ciliated cells (cc) and glandular cells (gc) in various stages of secretion. ×11,600

Fig. 20. Glandular cells with secretion granules from epithelial bar of the gill-chamber. Note, on the left, the regranulating glandular cell with broadened ergastoplasmic cisternae and a dense body (db). ×15,400

5). In the same zone the composition of the osmiophilic granules of the Golgi region as well as their function are still unknown.

The so-called iodine-binding zone. Zone VII has merited, in the last years, special interest because it has, according to the investigations of BARRINGTON and co-workers [3, 4], a iodine-binding capacity. Previously, it had been supposed that in larvae of Cephalochordata, Tunicata and Cyclostomata the function of the thyroid too is exerted by the endostyle, but the problem cannot be regarded at all as solved [1, 11, 18].

From morphological point of view, zone VII contains special cells bearing no resemblance to cells in other zones of the endostyle, nevertheless, they cannot yet be analogized, on the basis of electron micrographs, with cells in the thyroid of higher animals. In *Ciona intestinalis* there is little doubt that in the rough-surfaced endoplasmic reticulum of these cells some substance is produced, though, the cytoplasmic granules resemble rather the residual (lipofuscin) bodies than secretion granules.

It is remarkable that in the transitional zone of the endostyle and the gill-chamber (Fig. 1, VIII t) some cell-files have the same structure as the cells in zone VII; we mean, therefore, that in investigating the physiological role of zone VII, also these cells deserve attention.

Branchial bars. The glandular cells in the branchial bars are in conspicuously differing stages of secretion, therefore, they can actively function in feeding processes proceeding in the gill-chamber. Ciliated cells of the branchial bars probably promote the transport of the food particles as well as the mixing of the glandular secretion with food.

Remarks on the function of the endostyle and the gill-chamber

On the basis of an exclusively morphological study it is difficult to draw adequate conclusions for the function of the endostyle and the gill-chamber. The discovery of the topography and the structure of the various cell-types as well as the classification of the cells, though, enable us to studying the functional problems and several others, e.g., the following:

In the endostyle four glandular cell-types can clearly be differentiated: (1) mucus-producing cells in zone I, (2) glandular cells of zones II and IV which have the same structure and contain the same secretion granules, (3) cells of zone VI, (4) terminal cells of zone VII very resembling the glandular cells in the epithelial thickenings of the pharynx.

Cells of zone VII (so-called iodine-binding zone) probably have some secretion capacity, for the cisternae of their endoplasmic reticulum contain both a fine floccular substance and a few osmiophilic granules. However, the cytoplasmic granules cannot be marked off from the residual bodies.

It may be supposed, on the basis of the differences existing between structure and granules of the glandular cells, that the cells could produce various

substances which might have the following functions: paralyzing the living food (protozoa, planktons), sticking together the food particles and starting the digesting processes. As a consequence of this, the glands of the endostyle and the pharynx may be regarded as analogous with the salivary glands of higher animals as already suggested by others [11].

The glandular zones bear cilia and microvilli too, consequently, they cannot be rigidly separated from the so-called ciliated zones. The conspicuously regular arrangement of the microvilli suggests that also resorption processes take place in the endostyle and that the ciliated as well as the glandular cells equally participate in these. Also the complex vesicles observed in zones II and IV point to resorption processes.

Ciliated cells of the gill-chamber may act in transporting the food particles towards the esophagus and in mixing them with the secretion of the glands. The role of the extreme complex ciliated zones of the endostyle is all the more difficult to explain. Considering the localization of the ciliated zones and the orientation of the cilia we suppose that in the endostyle the ciliary movement promotes the transport of the secretion as well as its discharge in coordination with the functional requirements. During the respiratory movements the cilia probably prevent the glandular secretion of the endostyle from being diluted in respiratory water and from being removed through the gill-splits.

The terminal cells of zone VII of the endostyle and the ciliated cells of zone VIII are essentially identical with the cells in the epithelial thickenings of the gill-chamber and, therefore, these two zones cannot be taken, morphologically, for special components of the endostyle.

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DIE FEINSTRUKTUR DES KIEMENINNEREN UND DES ENDOSTYLS EINER ASCIDIENART *(CIONA INTESTINALIS)*

In der Wand des Kiemeninneren von *Ciona intestinalis* wurde die Feinstruktur des Endostyls und der Kiemenleisten elektronenmikroskopisch untersucht. Die Zone I des Endostyls läßt sich auf axiale und laterale Zellen unterteilen, beide Zellentypen entsprechen dem schleimerzeugenden Flimmerepithel. Die Zonen II und IV sind aus Drüsenzellen gleichen Typs aufgebaut, ihr Produkt ist ein granulärer Stoff unbekannter Natur. Die Zone IV besteht gleichfalls aus Drüsenzellen, auf deren apikalem Pol sich große Mengen eigenartiger Granuli anhäufen. Die Zonen III, V und VIII bestehen aus Flimmerepithel. An der Oberfläche der III. und V. Zone finden sich auch regelmäßig angeordnete Mikrozotten. Das endoplasmatische Retikulum von grober Oberfläche in den Plattenepithelzellen der Zone VII enthält eine feine flokkulierte Substanz und bisweilen osmiophile Körnchen. Im Zytoplasma dieser Zellen finden sich viele lipofuscinartige Einschlüsse. In den Epithelleisten des Kiemeninneren wurden mit Flimmern versehene und in unterschiedlichen Sekretionsphasen befindliche Drüsenzellen beobachtet. Einige Zellen der Zonen VII und VIII sind den Drüsenzellen der Epithelleisten des Kiemeninneren ähnlich.

МИКРОСТРУКТУРА ВНУТРЕННЕЙ ЧАСТИ ЖАБЕР И ЭНДОСТИЛЯ У ВИДА *CIONA INTESTINALIS*

В стенке внутренней части жабер *Ciona intestinalis* проводилось электронномикроскопическое изучение тонкой структуры клеток эндостиля и жаберных лепестков. Зона 1 эндостиля подразделяется на аксиальные и латеральные клетки; оба типа клеток соответствуют слизеобразующему мерцательному эпителию. II и IV зоны построены из желе-

зистых клеток одинакового типа, их продуктом является зернистое вещество неизвестной природы. Зона IV состоит также из железистых клеток, на апикальном полюсе которых накопляется значительное количество своеобразных зернышек. Зоны III и V и VIII состоят из мерцательных клеток. На поверхности зон III и V видны также равномерно распределенные микроворсинки. В клетках эпителия III-й зоны грубоповерхностная эндоплазматическая сетчатка содержит тонкое коагулированное вещество и иногда осмиофильные зернышки. В цитоплазме этих же клеток выявляемо множество липофусциноподобных включений. В эпителиальных пластинках внутренней части жабер наблюдались мерцательные клетки и железистые клетки, находящиеся в различных фазах секреции. Некоторые из клеток VII-й и VIII-й зон напоминают железистые клетки эпителиальных пластинок внутренней части жабер.

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STUDIES ON THE METABOLISM OF VIRUS-INFECTED PLANTS

VI. ASH CONTENT OF VIRUS-INFECTED AND HEALTHY PRIMARY LEAVES OF PHASEOLUS VULGARIS

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Synopsis

In Pinto beans infected by tobacco mosaic virus (TMV) or tobacco necrosis virus (TNV) the ash content of the uninfected leaves decreases while that of the infected leaves increases in the time between inoculation and the first appearance of symptoms. The more severe the infection, expressed in the number of local lesions, the greater are the deviations from the normal values. It is concluded, that these changes are caused by a flow of substances from the healthy to the infected parts of the plants.

Introduction

Virus-infected plants display, besides the characteristic symptoms, other changes in their respiration, amino acid, carbohydrate, and chlorophyll content [6] and enzyme activity [4]. However, in these regards there are no comparative data for the "infected" and the "uninfected" parts of the same plant. The questions arise:(1) how localized is a "localized" virus infection in fact?(2) does a TMV-infected Pinto-leaf react to the infection without influencing the uninfected parts of the plant? (3) if there is such an influence, how intensive is it, when does it start and end?

Material and method

Phaseolus vulgaris "Pinto" plants, 12—15 days old, grown in an ordinary greenhouse were decapitated. One group served as control, one was infected with undiluted, a third group with 100-fold diluted, inoculum. Inocula were prepared of lyophilized press saps of TMV-infected *Nicotiana tabacum* "Samsun" or TNV-infected *Phaseolus vulgaris* "Saxa". We inoculated without abrasive, using a flat ground glass rod. Thirty minutes later the inoculated leaves were thoroughly rinsed with tap water. One primary leaf of each plant was inoculated and marked. At fixed times subgroups of 10 plants were harvested. Inoculated and uninoculated leaves without petioles were weighed, dried at 110°C, weighed again, pulverized and 2 or 3 weighed samples were ashed at 850°C until constant weight, usually 2 hours. Samples were taken until symptoms appeared.

Results

Local lesions were counted 3 days after infection on about 20 plants (Tables 1, 2). Dry weight (in per cent of fresh weight) decreased in the uninoculated leaves in favour of the inoculated ones. The difference in dry weight

between inoculated and uninoculated leaves was the greatest shortly after inoculation and greater in plants inoculated with undiluted inoculum than in those inoculated with diluted inoculum.

Table 1

Ash weight in per cent of dry weight of TMV-inoculated and uninoculated primary leaves of Pinto beans

Averages for 4 experiments

Treatment of		sample taken hours after inoculation					mean of local lesions per leaf
plant	leaf	0	1	6	24	48	
control		14.73	13.53	13.82	13.55	14.66	
inoculated TMV-sap undiluted	inoculated		13.84	14.15	14.76	14.76	104.3
	uninoculated		13.47	13.25	13.85	13.88	
inoculated TMV-sap 100-fold diluted	inoculated		13.73	13.43	13.60	13.76	13.9
	uninoculated		13.22	13.33	13.42	13.60	

Table 2

Ash weight in per cent of dry weight of TNV-inoculated and uninoculated primary leaves of Pinto beans

Averages for 2 experiments

Treatment of		sample taken hours after inoculation					mean of local lesions per leaf
plant	leaf	0	1	6	24	48	
control		13.70	14.06	14.68	14.82	15.43	
inoculated TNV	inoculated		14.56	14.50	15.99	15.35	3.4
	uninoculated		14.46	14.04	14.77	15.12	
inoculated TNV 100-fold diluted	inoculated		14.24	13.13	14.22	14.45	0
	uninoculated		14.84	12.93	14.15	14.43	

Discussion

When after inoculation the infected tissue starts virus synthesis, it probably very soon exhausts the building blocks available, e.g., amino acids and nucleotides. So it will begin to synthesize new ones. This synthesis affords energy which has to be provided. It is to be expected that the uninfected cells around

the infected ones start sending building block as well as energy-supplying substances into the virus-synthesizing tissue [9]. Whether this transport is triggered by disturbed balance of substance distribution or by signal substances or hormone-like material sent out by the infected cells is an open question. It might be that in supplying material to the infected tissue, the surrounding uninfected tissue depletes itself of substances necessary for virus synthesis — so helping to block the further spreading of infection and becoming resistant to new infection, as observed by YARWOOD and ROSS [5, 8]. It was to be checked whether the influence of a virus infection is confined to the infected leaf or whether it extends to further parts of the plant. Table 1 shows clearly that, depending on the severity of infection, the uninoculated leaf receives a smaller supply of ash contents or that it sends some of its minerals to the infected leaf. Even the relatively weak infection by undiluted TNV inoculum increases ash content of the infected leaf, compared with the uninfected one. Since the differences in dry weight (in per cent of fresh weight) between infected and uninfected leaves are of about the same order, it might be supposed that not only minerals, but also organic metabolites are sent from the uninfected leaves into the infected one. This may or may not be a virus-specific phenomenon; EL KHALIFA and LIPPINCOTT [3] have reported that in crown-gall tumour bearing Pinto plants the healthy leaf is about 10% shorter and 20% lighter than the infected one. It seems that a virus infection markedly changes water relations inside the plant. FOLLMANN [1] reported increased cell sap concentrations and water intake of plant parts with symptoms due to various viruses. In local lesions produced by fungi HARVEY [2], using dyes, found higher transpiration than in the surrounding tissue. There, too, ash content increased. I found in attached as well as in detached Pinto leaves, infected with TMV or TNV, increased transpiration [7].

It might be concluded that a local virus infection increases the water and substance need of the infected tissue. That need is supplied by the surrounding tissue and by the other parts of the plant. Probably the increase in ash content is the greatest in the tissues displaying symptoms. So it might be expected that there the relative ash content is far higher than in the whole leaves examined in the present study. Nor does it seem improbable that in infected leaves there are zones with smaller ash content than in healthy ones, due to translocation from there towards the symptoms forming places.

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UNTERSUCHUNGEN ÜBER DEN STOFFWECHSEL VIRUSKRANKER PFLANZEN

In *Phaseolus vulgaris* L. var. "Pinto" nahm der Aschegehalt (bezogen auf Trockengewicht) der mit TMV bzw. TNV infizierten Blätter zu, während der der nicht infizierten Blätter abnahm. Je stärker die Infektion war — gemessen an der Zahl der Lokalläsionen — um so größer war die Zunahme des Aschegehaltes der infizierten und die Abnahme des Aschegehaltes der nicht infizierten Blätter. Es wird vermutet, daß diese Veränderungen das Ergebnis eines Stofftransportes aus den gesunden in die infizierten Teile der Pflanze sind.

ИССЛЕДОВАНИЕ ОБМЕНА ВЕЩЕСТВ У РАСТЕНИЙ, ЗАРАЖЕННЫХ ВИРУСАМИ

VI. Содержание золы в зараженных вирусами и в здоровых первичных листьях растений Phaseolus vulgaris

В листьях растений *Phaseolus vulgaris* L. var. "Pinto", зараженных TMV и TNV, содержание золы (в переводе на сухой вес) повышается, тогда как в незараженных листьях оно уменьшается. Чем тяжелее инфекция — на основе числа местных повреждений — тем значительнее и повышение концентрации золы в зараженных и уменьшение содержания золы в незараженных листьях. Полагается, что эти изменения вызываются транспортом веществ из здоровых частьях в зараженные органы растения.

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DAMAGING EFFECT OF NEONATAL ANDROGEN TREATMENT ON THE PRODUCTION OF GONADOTROPHIC HORMONES

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Synopsis

The paper deals in detail with the pathophysiological effect of androgenization on the hypothalamic structures and points to the contradictory reports published on this subject. The hypothalamus of newborn, genetically male rats was exposed to four kinds of differently strong androgenic effect. The level of gonadotrophic hormone (GTH), produced by the pituitary gland of the adult animals in the four groups was studied by the method of parabiosis. The ovaries and uterus of control and androgenized partners served as detectors. Parabiosis is claimed to be the most suitable and most sensitive method since it eliminates all the feed-back effects. Rats treated with large doses of testosterone immediately after birth were incapable of an increased GTH production, whereas the same treatment on the fifth day after birth did not interfere with the synthesis of GTH. Relying on the results of the present and earlier experiments the authors assume that androgen treatment, besides having a desensitizing effect on the preoptic area of the hypothalamus, damages those structures in the hypophyseotrophic area which are responsible for the basic GTH secretion.

Introduction

It is generally recognized that "masculinization" of the hypothalamus in newborn rats is due to androgen produced by the infantile testicles during the so-called critical period [2, 4, 7, 13, 14, 17, 22]. According to HARRIS and LEVINE [18] and BUSCHBECK [5] the hypothalamus is originally of the female type and in the absence of the testosterone it retains this character. LADOSKY [26], on the other hand, claims that ovarian hormones, too, are indispensable for the development of a normal female-type hypothalamo-hypophyseal system. Neonatal androgen treatment induces, according to BARRACLOUGH and GORSKI [4] and GORSKI and WAGNER [14], a functional disturbance of the structures existing in the preoptic-suprachiasmatic area. FLERKÓ [10] points to this area as the site of the neurons of the "LH-trigger" and "FSH control" mechanisms. There is no doubt that biochemical and morphological phenomena pointing to functional disorders have been demonstrated in the anterior part of the hypothalamus.

DÖCKE and KOLOCZEK [6] observed genetic dimorphism in the anterior hypothalamic nuclei. Neonatal testosterone treatment prevented cyclic fluctuations, but failed to prevent dimorphism. Studying the glycolytic and oxi-

dative metabolism of the hypothalamus, MOGUILEVSKY and RUBINSTEIN [27] found that androgenization increased the oxidative activity in the anterior hypothalamus of female rats.

There are two contradictory hypotheses regarding the *true nature* of the effect produced by neonatal androgen treatment. FLERKÓ, relying on a long series of experiments [9, 10, 11, 31, 32, 33, 37, 38], suggests that androgen *desensitizes* the hypothalamic neural elements sensitive to sex steroids. As a result, elevation of the steroid level in the serum elicits no response, i.e. the cyclic outflow of the LH and the decrease in FSH production.

MORRISON and JOHNSON [30] are of the opposite opinion. They maintain that the hypothalamo-hypophyseal system of androgenized male rats becomes *more sensitive* to androgen. Adult male rats, castrated and androgenized after birth (hypothalamus of the male type) required a smaller dose of testosterone for the inhibition of GTH secretion than did neonatally non-castrated animals. In contrast, PETRUSZ and FLERKÓ [31] inhibited GTH secretion by exogenously administered oestrogen more markedly in male rats castrated on the second postnatal day (hypothalamus of the female type) than in males castrated after 60 days. In the latter group the physiological amount of androgen produced by the testicles desensitized the hypothalamus. Oestrogen inhibits compensatory ovarian hypertrophy to a lesser degree in androgenized females than in the control animals [33].

The clash of the two hypotheses cannot be explained by methodical differences (parabiosis or transplantation and compensatory hypertrophy) or those between the injected steroids (testosterone or oestradiol). Moreover, no sex specificity could be demonstrated in respect of the GTH-inhibiting action of steroids [36].

However, the findings of MORRISON and JOHNSON [30] admit of an alternative interpretation. Two observations are to be taken into consideration: (a) an androgen given on the first day after birth exerts an effect which is freakly different from the effect exerted by the same hormone if administered on the fifth day; (b) the testosterone administered on the first day effects also the neural structures [25] which, situated in the hypophyseotrophic area described by HALÁSZ and PUPP [15, 16] maintain the secretion of GTH.

The present experiments had the purpose to subject to a quantitative analysis the GTH secretion of animals whose hypothalamus was exposed to *four differently* strong androgenic effects, and in whom feed-back effects (the mechanism whereof is still somewhat obscure) were eliminated. The results of the experiments were expected to resolve the contradiction between the respective hypotheses of FLERKÓ and MORRISON—JOHNSON and to offer, moreover, a wider interpretation of "androgenization".

Material and method

Inbred white rats of the strain "R-Amsterdam, Hungary", kept on standard diet and under equal condition, were used. Some of the newborn littermate male animals were castrated in ether anaesthesia, within the first 12 hours following birth, others on the fifth day of postnatal life. A dose of 500 μg testosterone phenylpropionate (Retandrol, Richter, Budapest) dissolved in 0.1 ml oil, was subcutaneously injected into 50% of the animals at the time of castration.

Each of the castrated and castrated + androgenized males was parabiotically associated with one of its female littermates of the same body weight between the 42nd and the 50th days. Most of the females ("detectors") were controls, the others had been androgenized on the fifth day. Parabiotic union was effected by FLERKÓ's method [8].

The pairs were separated in ether narcosis on the 12th postoperative day; after determining their body weight we decapitated the animals. We weighed the hypophysis, the thyroid, the adrenal glands and the ovaries on torsion balance ($\pm$ 0.2 mg) and fixed the organs in 4% formalin for the purposes of later histological analysis. Certain accessory genital organs (uterus, seminal vesicle) were likewise weighed. Fourteen pairs died in the course of parabiosis; the respective data were disregarded at the evaluation of results.

Table 1

Ovarian and uterine weight of the female parabiotic partners of male animals castrated on the 1st, 5th or the 50th day

	Detector ♀	Partner ♂	Number of pairs	Body weight at dissection (g)	Weight of ovary (g)	Weight of uterus (g)
1.	C ♀	1 CC ♂	8	113.6 $\pm$ 5.2*	181.8 $\pm$ 25.1	270.2 $\pm$ 23.0
2.	C ♀	1 CA ♂	10	111.5 $\pm$ 2.6	53.4 $\pm$ 4.6	168.9 $\pm$ 15.2
3.	C ♀	5 CC ♂	11	108.8 $\pm$ 1.0	156.9 $\pm$ 21.0	264.1 $\pm$ 10.8
4.	C ♀	5 CA ♂	8	107.7 $\pm$ 3.2	171.0 $\pm$ 27.1	298.5 $\pm$ 18.1
5.°	C ♀	50 CC ♂	11	123.3 $\pm$ 5.2	131.3 $\pm$ 13.1	263.0 $\pm$ 28.0
6.°	C ♀	50 C 1 A ♂	7	117.6 $\pm$ 5.0	46.6 $\pm$ 3.9	144.0 $\pm$ 19.0
7.°	C ♀	—	11	124.5 $\pm$ 5.0	39.2 $\pm$ 2.1	193.0 $\pm$ 22.0
8.	5 A ♀	5 CC ♂	4	116.5 $\pm$ 2.5	139.5 $\pm$ 20.8	210.5 $\pm$ 10.5
9.	5 A ♀	5 CA ♂	5	119.8 $\pm$ 4.9	201.1 $\pm$ 43.1	233.4 $\pm$ 16.6

Ovary **: 1—2 $P < 0.01$; 2—4 $P < 0.01$; 2—3 $P < 0.01$; 5—6 $P < 0.01$;

Uterus **: 1—2 $P < 0.01$; 2—4 $P < 0.01$; 2—3 $P < 0.01$; 5—6 $P < 0.01$;

* Standard deviation ** Probability ° Data obtained from another strain used in an earlier study [25].

The following symbols were used to designate the experimental groups in text and Table:

1 CC ♂ = control males castrated on the first day;
1 CA ♂ = males castrated and androgenized on the first day;
5 CC ♂ = control males castrated on the fifth day;
5 CA ♂ = males castrated and androgenized on the fifth day;
50 CC ♂ = control males castrated on the 50th day;
50 C1A ♂ = males androgenized on the first, castrated on the 50th day;
5A ♀ = females androgenized on the fifth day;
C ♀ = control females

Data for groups 50 CC ♂, 50 C 1 A ♂ and the individual animals C♀, taken from an earlier work, are included in Table 1 for the facilitation of comparisons [25]. Student's "t" test was used for statistical analysis [23].

Results and discussion

As a consequence of postnatal operation and hormonal treatment the hypothalamus of genetically male rats in the four groups were exposed to different testosterone effects. The hypothalamus of the 1 CC ♂ animals remained of the "female type" since the testicles, having been removed immediately after birth, were not able to produce androgen during the five days of the "critical period", whereas the hypothalamus of the 1 CA ♂ animals received the maximal androgenic effect. On the hypothalamus of the animals 5 CC ♂ the physiological androgen, i.e., that produced by infantile testicles acted during five days whereafter the androgenic action was stopped by castration. In the case of animals 5 CA ♂, the physiological androgenic effect of the first five days was followed by super-maximal action. The other possible variations (e.g. castration on the first and androgenization on the 2nd to 5th day) would not have provided additional information. The degree of possible intrauterine androgenic effect was the same in all groups.

By the castration the peripheral component of the feed-back mechanism was eliminated. It was therefore possible to induce the maximum GTH production (secretion + release) by the hypophysis so that the degree of hypophyseal GTH production became a reliable indicator of the physiological condition of the hypothalamus.

Convincing data have indicated parabiosis as the best method for the determination of GTH production [19, 20, 21, 36]. Determination at a given time of hypophyseal [3, 28] or blood [34] FSH or LH, admits of no conclusion to the prolonged production of GTH by the pituitary gland. Considering that the GTH produced by the hypophysis of the control female (C ♀) and the individual variations in the number of corpora lutea and follicles in its ovaries might be disturbing factors, we used also 5 A ♀ animals as detectors since the ovaries of androgenized females produce no corpora lutea. By employing the method of parabiosis it is possible to follow changes in the weight of both the ovary and the uterus as indicators of ovarian hormone production.

Table 1 shows the ovarian and uterine weights of animals C ♀ and A ♀. These weights in the partners of 1 CA ♂ were the same as the corresponding data observed in the individual C ♀ and the 50 C 1 A ♂ partners. The negligible degree of hypertrophy justifies the conclusion that a large dose of androgen, if administered on the first day, prevents any increase in the GTH production. Such interference with GTH production was not observed in the animals 5 CC ♂, 5 CA ♂ and 50 CC ♂. It follows that:

(1) no matter whether it acts over five or fifty days, the physiological amount of androgen produced by the testicles does not induce changes sufficient to prevent increased GTH production following castration;

(2) a high dose of testosterone, causing serious disturbance if given on the first day, is completely ineffective if administered on the fifth day after birth.

The present results and those of earlier experiments [25] lead to the assumption that androgenization has (apart from its peripherial effect) a *double central effect*. Besides *desensitizing* the neural elements of the anterior hypothalamus which are otherwise sensitive to sex hormones [10] and thus turning the hypothalamus into the masculine type, it *damages* those neural structures of the hypophyseotrophic area which are responsible for basic GTH secretion. "Damage" in this sense means reduction in the productivity of the GTH-releasing factors. Androgenization on the fifth day either fails to cause such "damage" (this is less probable) or, else, the damage is but partial: it does not completely paralyse the functional capacity of the hypophyseotrophic area.

In the rat hypothalamus an increasing number of neurones undergo irreversible spontaneous changes during the postnatal days, and consequently, become insensitive to the damaging effect of even large doses of testosterone. The "irreversible spontaneous change" may, e.g., mean that the hypothalamic nerve cells are no longer capable of androgen uptake. This view is supported by the findings of ARAI and GORSKI [1] as also by WOLLMAN and HAMILTON [39] who succeeded in preventing androgenization by pentobarbital or an anti-androgenic substance.

How is it then possible to interpret the suggestive results of — say — MORRISON and JOHNSON referred to in the introduction? They castrated animals immediately after birth, and androgenized them on the fifth day. The hypothalamus was not exposed to androgenic action during the interval of four days. The large dose of testosterone (2.5 mg) desensitized the sex-steroid sensitive structures of the anterior hypothalamus (development of "male type"). However, of the neurones responsible for the basic secretion of GTH only those were damaged which had *not* undergone an irreversible spontaneous change in the past four days. The unimpaired structures were still capable of maintaining GTH production at an adequate level, though as small amount of exogenous testosterone as introduced into the organism of 1 C 5 A ♂ animals, was able to interfere with the mechanism which regulates GTH production. The observed phenomenon did not, therefore, consist in a further sensitization of the testosterone-sensitive hypothalamic elements but we believe that these elements are injured and consequently, a part of them becomes inactive. The capacity of the still active structures is, then, reduced by otherwise even ineffective inhibition, and they become unable to respond to the (presumably likewise weak) stimuli coming from the anterior hypothalamus

Androgenization, induced on the first day, reduces the power of the hypophyseotrophic area to secrete GTH so much as to remain practically unable to grow stronger even after castration.

This hypothesis does not explain the phenomena observed in connection with the transplantation or/and unilateral ovariectomy. We think, however,

that the intensity of GTH production cannot be estimated as long as feed-back effects cannot be excluded. The work of KOVÁCS [24] deals very thoroughly with histological changes occurring in the ovary transplanted to the spleen of androgenized rats. KOVÁCS — agreeing in this respect with the findings of MAYER and THEVENOT-DULUC [29], ROY and co-workers [35] and LADOSKY [26] — observed follicular growth and *invariably* registered luteinization in the intralienal ovary of castrated rats. On the other hand, ovaries transplanted subcutaneously show luteinization only in control females or control male animals castrated before the fifth day of life [14]. Corpora lutea never develop spontaneously in the extralienal ovary of androgenized females and males, nor in that of male animals castrated after the fifth day. In our opinion subcutaneous transplantation is the nearest approach to in situ conditions, although unelucidated feed-back mechanisms interfere with hypophyseal GTH production in cases of both extralienal and intralienal grafting. No feed-back mechanisms are present in parabionts.

The statements made in the foregoing apply also to results obtained from unilateral castration and compensatory hypertophy, further to the conclusions following from these results. It has already been demonstrated [12] that the compensatory ovarian hypertrophy of androgenized animals depends on the time of testosterone treatment and also on the age at which the animals are unilaterally castrated. Compensatory gonadal hypertrophy is more pronounced in 60-day-old female animals androgenized on the first day than in the controls, while the situation is inverse at the age of 140 days. Contradictory — though not incorrect — data may give rise to diametrically opposed theories.

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DIE SCHÄDIGENDE WIRKUNG DER ANDROGENBEHANDLUNG IM NEUGEBORENENALTER AUF DIE GONADOTROPHORMON-PRODUKTION

In der Arbeit wird die pathophysiologische Wirkung der Androgenverabreichung auf die hypothalamischen Strukturen behandelt und auf die widersprechenden einschlägigen Mitteilungen verwiesen. Der Hypothalamus neugeborener, genetisch männlicher Ratten wurde vier verschieden starken androgenen Wirkungen ausgesetzt. Der Gonadotrophormon-(GTH)-Spiegel, den die Hypophysen der erwachsenen Tiere der vier Gruppen produziert hatten, wurde mittels Parabiose ermittelt. Zur Aufklärung dienten die Ovarien und der Uterus der Kontrolltiere und der androgenisierten Partner. Die Parabiose wird als eine sehr geeignete und empfindliche Methode betrachtet, da sie sämtliche feedback-Effekte eliminiert. Die unmittelbar nach der Geburt mit großen Testosterongaben behandelten Ratten waren zu einer erhöhten GTH-Produktion unfähig, während die gleiche Behandlung am 5. Tage nach der Geburt keine Herabsetzung der GTH-Synthese bewirkt hatte. Gestützt auf die Ergebnisse der gegenwärtigen sowie früherer Experimente folgern die Verfasser, daß die Androgenbehandlung neben einem Desensivitierungseffekt auf die präoptische Zone des Hypothalamus jene Strukturen in der hypophyseotropischen Zone schädigt, die für die Grundsekretion der gonadotropen Hormone verantwortlich sind.

НАРУШЕНИЕ ОБРАЗОВАНИЯ ГОНАДОТРОПНЫХ ГОРМОНОВ ВСЛЕДСТВИЕ ДАЧИ АНДРОГЕННОГО ГОРМОНА В ПЕРИОД НОВОРОЖДЕННОСТИ

Дается подробное обсуждение патофизиологического эффекта дачи андрогенного гормона на гипоталамические структуры и указывается на противоречивые данные, имеющиеся в литературе по этому вопросу. Гипоталамус новорожденных, генетически мужских крыс, распределенных в четыре группы, были подвергнуты андро генным влияниям различной интенсивности. Уровень гонадотропных гормонов (ГТГ), образуемых гипофизом взрослых животных четырех групп, изучался методом парабиоза. Для выявления служили яичники и матки контрольных крыс и их партнеров, получивших андрогенный гормон. Парабиоз рассматривается как очень подходящий и чувствительный метод, так как элиминирует все эффекты обратной связи. Крысы, получившие большие дозы тестостерона немедленно после рождения, не были способными к повышенному синтезу ГТГ, тогда как дача того же количества гормона на пятый день после рождения не вызвала нарушения синтеза ГТГ. На основе результата настоящих и прежних экспериментов авторы полагают, дач чтоа андрогенов, помимо десенситивизации преоптической зоны гипоталамуса повреждает те структуры гипофизеотропической зоны, которые ответственны за основную секрецию ГТГ.

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STUDY OF FLORAL ANATOMY OF THE TRIBE JUSTICIEAE (ACANTHACEAE) WITH AN AIM OF TAXONOMICAL INTERPRETATIONS

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Synopsis

From the study of the floral anatomy of some species of *Barlerieae*, *Andrographideae* and *Eujusticieae* of the tribe *Justicieae* of BENTHAM and HOOKER, it has become clear that *Barleria* is more primitive than others in the tribe and its distinctive features support the placing of *Barlerieae* in a taxon different from the *Andrographideae* and *Eujusticieae* of ENGLER and DIELS.

Introduction

The tribe *Justicieae* of BENTHAM and HOOKER [1] of the family Acanthaceae has been studied from cytological and anatomical bases [2, 3]. There is a controversy regarding the position of *Barleria*. According to BENTHAM and HOOKER, the tribe *Justicieae* includes the subtribes *Barlerieae*, *Andrographideae* and *Eujusticieae*. But according to ENGLER and DIELS [4] the *Barlerieae* are under *Contortae* near *Ruellieae*, while *Justicieae* and *Andrographideae* are under *Imbricatae*, a different taxon.

Both cytological and anatomical studies have led to the conclusion that the genus *Barleria* represents the most primitive status among the studied genera of the *Justicieae* of BENTHAM and HOOKER. Further, their characters are quite distinct from those of *Eujusticieae* and *Andrographideae*. The present attempt is to gather some information regarding the vascular supplies in floral organs, which may help in solving the controversy.

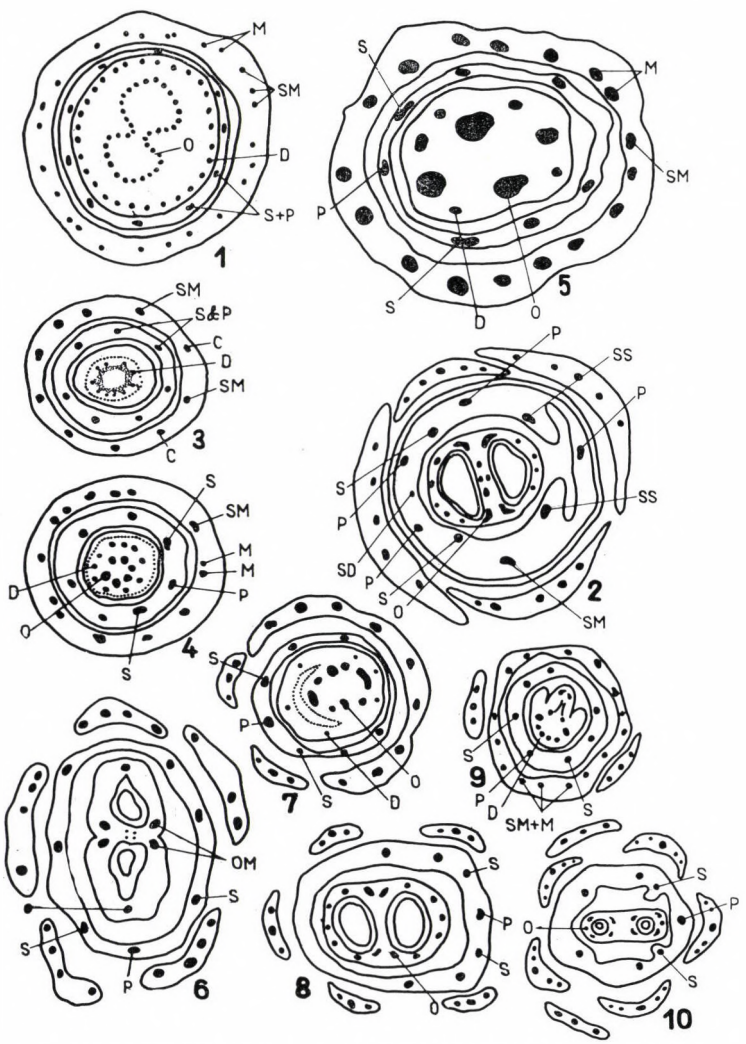
Material and method

The following species were collected from Calcutta and suburbs:
Subtribe *Barlerieae: Barleria prionitis* L.
Subtribe *Andrographideae: Andrographis paniculata* NEES.
Subtribe *Eujusticieae: Ecbolium linneanum* KURZ., *Justicia gendarussa* BURM., *Adhatoda vasica* NEES.

Flower buds at fully differentiated condition were fixed in formalin-propiono-alcohol [5]. Paraffin blocks were cut at 14 μ, and stained with crystal violet.

Observations

Barleria prionitis L. (Fig. 11). The calyx traces originate from the stele from ten separate gaps, proceed obliquely towards the periphery of the receptacle, up to about 90—100 μ, after which the traces are approximately perpendicular. Of the ten traces, the four midrib-dorsal traces (of four calyx lobes) are found to produce three branches almost immediately within the receptacle.



Figs 1—10. Diagrams showing the distribution of vascular traces in floral members in T. S. *1, 2 — Barleria prionitis* L.; *3, 4, — Andrographis paniculata* NEES; *5, 6 — Ecbolium linneanum* KURZ.; *7, 8 — Justicia gendarussa* BURM.; *9, 10 — Adhatoda vasica* NEES. (SM = sepal-midrib trace or its branches; M = sepal-margin trace; S = stamen trace; P = petal trace; D = disc trace; O = ovary trace; OM = ovary-margin traces; SS = sterile stamen traces; SD = stamen trace discontinued)

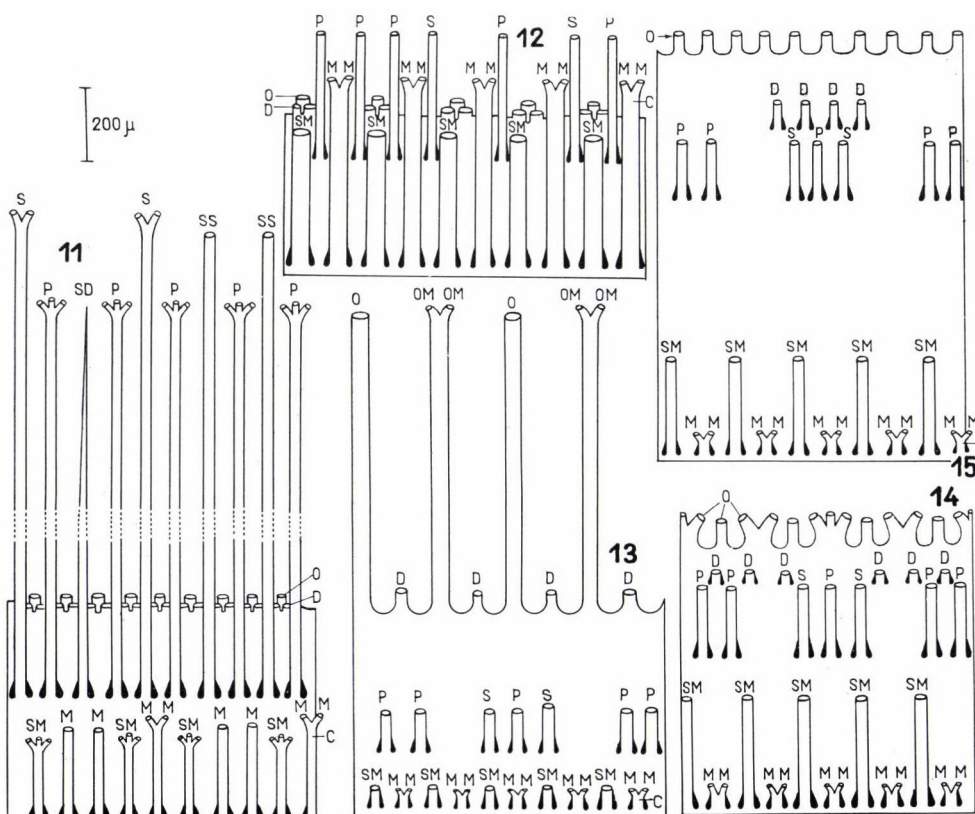
Of the remaining six bundles, four supply the four margins, and two act as commissural bundles, each of which divide and supply the adjacent margins. Thus all the eight margins are supplied, four by original traces and four by division of two. Further branching is accomplished in the calyx tube (Fig. 1). Five corolla traces and five stamen traces (Figs 1 and 2) originate after a long distance of about 270 μ, above the origin of calyx traces (Fig. 11). Stamen traces and corolla traces remain in the corolla tube. Corolla traces branch into three each and supply the five subequal imbricately arranged lobes. One of the five stamen traces narrows and discontinues. Stamens are four in number, two fertile and two sterile. The fertile stamens are supplied by three branches of a staminal trace, formed just below the anther. The sterile ones remain massive and traces for them do not branch. A large number of disc traces originate from the central stele at a level of about 200 μ above the origin of corolla and stamen traces (Fig. 1), move to the periphery of the massive disc-base and gradually form an annular disc, very high, above 600 μ. The carpellary traces orient themselves into a bilobed structure, gradually organize into four central bundles and six major carpellary bundles, each of the two ovaries having three (one dorsal and two marginal). The central bundles separate on the sides of the transmission tissue and other bundles continue up to the stigma.

Andrographis paniculata NEES. (Fig. 12). Ten calyx traces proceed perpendicularly in the receptacle without branching. Out of these ten bundles five are sepal midrib traces, the other five are commissural traces for supplying two adjacent sepal margins. Each of the commissural traces divides into two, about 500 μ above the origin of bundles. Unlike *Barleria* sp., the sepal midrib traces do not divide, even in the calyx lobes. Seven traces (five for corrolla and two for stamens) originate at about 250 μ above the origin of calyx traces (Figs 3 and 4). The two staminal traces take an inward position in the corolla tube and supply the two filaments formed much below the corolla throat. The five corolla traces remain unbranched until they reach the four lobes. The staminal traces produce lateral branches just below the 2-celled anthers. The low and massive disc is supplied by ten traces originated from the stele. The basal non-vascular portion of the disc remains isolated from the outside tissue and more or less encased within a unilayered tissue of thick-walled cells, probably lignified (Fig. 3). This layer may be a protective structure for checking transpiration from the inner bundles supplying the carpels. At a higher level this layer moves outwards and the ten disc traces come out from the stele. No annulus formed by the disc has been found. After the differentiation of the disc, the inner bundles organize into six ovarian traces (Fig. 4), three (one dorsal and two marginal) supplying each of the two. The bifid style bears at the tip a bilobed stigma, each of which is supplied by a fusion bundle of three.

Ecbolium linneanum KURZ. (Fig. 13). Ten bundles, five sepal midrib traces and five commissural marginal traces of sepals, originate from the stele at

different levels. The commissural traces divide almost immediately into two marginal traces resulting in a total of 15 traces in the calyx tube, which remain undivided in the lobes. About 140 μ above the origin of calyx traces, the seven traces, five for corolla and two for stamens come out from the stele (Figs 5 and 6). The petal traces remain unbranched for a long distances. About 400 μ above the origin of corolla and stamen traces, about four traces are noticed in the massive disc, which is a complete annulus. Four bundles for the ovaries (Fig. 5), two dorsal to each ovary and two commissural marginal between the ovaries are found distinct almost at the same level. Two commissural marginal traces of the ovaries branch into two marginal traces at 840 μ above the origin (Fig. 6). They fuse again at the base of the style, 1,260 μ above the level of branching.

Justicia gendarussa BURM. (Fig. 14). The calyx is supplied by ten bundles (five commissural marginal and five sepal midrib). The commissural bundles



Figs 11—15. Diagrammatic representation of vascular traces in flowers in longitudinal direction (as if the vascular stele has been split longitudinally and spread open). *11 — Barleria prionitis* L.; *12 — Andrographis paniculata* NEES.; *13 — Ecbolium linneanum* KURZ.; *14 — Justicia gendarussa* BURM.; *15 — Adhatoda vasica* NEES.
(Abbreviations see Figs 1—10)

begin to divide even below the formation of other calyx traces. The outer commissural trace is dividing first. The midrib traces remain undivided even in the lobes. The calyx is two-lipped (lobes — dors. 2 + vent. 3). Seven traces, five for corolla and two for stamens, originate at a level of more than 400 μ above the origin of calyx traces (Figs 7 and 8). The dorsal trace comes out much below the others. The corolla is two-lipped (adaxial and abaxial), supplied by three and two traces, respectively. The two stamens are supplied by two traces, each of which branches into three below the 2-celled anthers. The massive short disc (horse-shoe-shaped in T. S.) is supplied by a few (about six) vascular traces (Fig. 7). It incompletely covers the ovary on the adaxial and lateral sides, the dorsal side remaining uncovered. The carpels are at first supplied by eight bundles (Fig. 7), three situated at the dorsal side of each of the ovaries and two between the two ovaries, probably acting as two commissural marginal traces common to both (Fig. 14). These traces divide at an upper level, where each of the ovaries contains two marginal bundles and five other bundles (Fig. 8).

Adhatoda vasica NEES (Fig. 15). Ten calyx traces (five sepal midrib and five commissural marginal) originate from the central stele. The abaxial trace is a commissural one, which divides immediately after its origin, others at successive levels. The marginal traces divide in the lobe. The calyx is five-lobed, arranged valvately in the bud. Seven traces (five for corolla and two for stamens) originate about 700 μ above the origin of calyx traces (Figs 9 and 10). The corolla is two-lipped, the adaxial of three and the abaxial of two petals. They are supplied by three and two traces, respectively. The staminal traces coming out of the corolla tube divide into three below the anthers, which are two-celled and apiculate. The disc is, similar to *Justicia*, an incomplete ring, covering the ovary only on the adaxial and lateral sides. Disc bundles are very few in number (3—5). Each of the ovaries is supplied by five traces, two marginal, one dorsal and two lateral (Fig. 10).

Discussion

Barleria prionitis (*Barlerieae*) is distinct from all the rest of the species studied here, in the vascular supplies to floral parts. The distinctive features are (1) oblique direction of the calyx traces, (2) presence of both commissural marginal and direct marginal traces for sepals, (3) formation of a very high annular disc supplied by a large number of bundles, (4) five staminal and five petalline traces, (5) formation of four opposite sepal lobes and five imbricate petal lobes, (6) formation of four stamens, two of which are fertile and 4-celled in the early stage, (7) division of petal-midrib traces into three. *Andrographis paniculata (Andrographideae)* is distinguished from *Barleria prionitis* by (1) perpendicular calyx traces, (2) the absence of direct marginal traces for sepals and the forma-

tion of regular commissural marginal traces, (3) disc not annular and supplied by about ten traces, (4) formation of five petalline and two staminal traces, (5) five sepal lobes and four petal lobes and (6) 2-celled anthers. This species is distinguished from the three species of *Eujusticieae* by (1) the absence of annular disc, (2) regular, valvate sepal lobes, (3) four corolla lobes. *Ecbolium linneanum*, *Justicia gendarussa* and *Adhatoda vasica* (all *Eujusticieae)* can be distinguished from *Barleria prionitis* by (1) perpendicular calyx traces, (2) absence of direct marginal traces and presence of regularly arranged alternate commissural strands and midrib traces for sepals, (3) absence of division of the sepal midrib traces, (4) formation of five calyx lobes and two-lipped corolla, (5) formation of five corolla traces and two staminal traces, (6) annular disc supplied by a few (3—6) traces. These three species can be distinguished from *Andrographis paniculata* by the (1) annular disc supplied by fewer traces, (2) larger number of ovarian traces. So it is evident that the genera representing different subtribes have distinctive features, probably characteristic of those subtribes.

It becomes clear that *Andrographis*, *Ecbolium*, *Adhatoda* and *Justicia* have a well-organized and simplified vascular system in their flowers, while *Barleria* has a clumsier type of calyx trace organization with a tendency of early branching. Regular pentamerous condition in the vascular traces of stamens has been still retained in *Barleria*. The vascular traces seem to show some gradual reduction in number with the advancement of the species. Therefore, *Barleria prionitis* represents no doubt the most primitive condition. The others show different stages of advancement.

The position of *Barleria*, which is a controversial one, can easily be settled from the study of the floral anatomy. It has already been stated that *B. prionitis* represents so many characters distinguishing it from *Andrographideae* and *Eujusticieae* that it seems quite justified to place this genus in a separate tribe outside the *Justicieae* of BENTHAM and HOOKER [1]. As was concluded from the cytological study and from the investigation of secondary xylem [2, 3], the present work also emphatically supports the placement of the *Barleriae* in a different taxon by ENGLER and DIELS [4].

From the study of the graphical representation of floral vasculature, it is seen that in the *Eujusticieae*, *Ecbolium linneanum* has the shortest average internodes of the floral axis, *Adhatoda vasica* the longest and *Justicia gendarussa* an intermediate. From the study of xylem it has been ascertained that *E. linneanum* is the most primitive and *A. vasica* the most advanced species. The internode length may, therefore, be related to evolution.

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UNTERSUCHUNG DER PFLANZENANATOMIE DES TRIBUS *JUSTICIEAE* (ACANTHACEAE) ZWECKS TAXONOMISCHER EINORDNUNG

Aus der Untersuchung der Pflanzenanatomie einiger Arten der Gattungen *Barlerieae*, *Andrographideae* und *Eujusticieae* des Tribus *Justicieae* von BENTHAM und HOOKER ging hervor, daß die Arten der Gattung *Barleria* primitiver sind als die der anderen Gattungen dieses Tribus. Ihre Unterscheidungsmerkmale motivieren die Einordnung der Gattung *Barleriae* in ein anderes Taxon als die Gattungen *Andrographideae* und *Eujusticieae* von ENGLER und DIELS (1936).

ИЗУЧЕНИЕ АНАТОМИИ ЦВЕТКОВ ТРИБУСА *JUSTICIEAE* В ЦЕЛЯХ ОПРЕДЕЛЕНИЯ ИХ ТАКСОНОМИЧЕСКОЙ ПРИНАДЛЕЖНОСТИ

На основе изучения анатомии цветков некоторых видов родов *Barlerieae*, *Andrographideae Eujusticieae* трибуса *Justicieae Бентхама* и *Хукер* стало известным, что виды рода *Barleria* являются более примитивными чем виды остальных родов этого трибуса. Их отличительные признаки мотивируют причисление рода *Barlerieae* в другой таксон, чем роды *Andrographideae* и *Eujusticieae Энглера* и *Дильса* (1936).

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AN IRON-ALUM METHOD FOR ELECTIVE DEMONSTRATION OF ACID MUCOPOLYSACCHARIDES. II

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Synopsis

An iron-alum method was evolved for the elective demonstration of acid mucopolysaccharides, and its chemical mechanism has been studied. The elective staining was found to be due to two factors. First, to the blocking action of the iron ions which form stable bonds with phosphate and carboxyl ions, second, to the more loose linkage between iron ions and sulphate groups which — according to the principle of competitive antagonism — enables acid mucopolysaccharides to bind the molecules of basic dyes. It was further found that, in a stable form, the protein component of the chondroprotein and mucoprotein is likewise able to bind iron ions, and that it is presumably involved in the mechanism through which dye is bound by intact chondro-muco-protein.

Introduction

A new method for the demonstration of acid mucopolysaccharides was described in Part I of this series. Pretreated with iron-alum, structures containing acid mucopolysaccharides stain electively with certain basic dyes. Concerning the chemical background of the elective staining we arrived at the following suggestions:

(1) For the elective staining the blocking effect of iron hydroxide ions is chiefly responsible, these inhibit the binding of basic dyes in all tissues except those containing acid mucopolysaccharides.

(2) That structures contanining acid mucopolysaccharides are stained despite the binding of iron is due to a competitive antagonism between the ions of basic dyes and the iron hydroxide ions. Being the linkage of the latter to the anions of the acid mucopolysaccharides weaker, they are displaced by the former.

The present investigations had the aim to check these suppositions and to make an attempt to elucidate the chemical mechanism of our procedure.

Material and method

Cartilage seemed to be the most suitable for the present purposes. It is available in abundance, contains a large amount of acid mucopolysaccharides, and both its structure and chemistry are fairly well known. We, accordingly, prepared sections of 5 to 7 μ thickness from the larynx and costal cartilage of white rats, fixed them in Carnoy's fluid and embedded in paraffin.

Acid mucopolysaccharide-containing structures were stained with the dyes described in Part I of this series of publications [3].

To determine the ions of iron hydroxide we transformed them to iron sulphide by adding ammonium sulphide, and to ferric ferrocyanide (Prussian blue) by adding potassium ferrocyanide [5].

The role played by the components of the acid mucopolysaccharide complex in the mechanism of staining was studied by the following methods:

(1) Methylation with hydrochloric methanol at 60°C for six hours [6].

(2) Blocking of polyanions by a 1% solution of cetyl pyridinium bromide (CPB) at 30°C for 24 hours [9].

(3) Digestion with a 2 mg/ml aqueous solution of hyaluronidase (pH = 5.4) for 24 hours [7].

(4) The iron-binding capacity of the various tissue proteins was studied by digesting at 37°C for two hours in a solution containing 1 mg/ml trypsin in phosphate buffer (pH = 8.9) [6].

(5) To study the role of the protein component of the mucopolysaccharide complex in binding iron, materials were digested in a 0.2% aqueous solution of papain (pH = 6.5) at 55°C for 30, 60, 120 and 240 minutes [4].

(6) Sulphation with a 1 : 1 mixture of concentrated sulphuric acid and ether for 10 minutes [6].

Also combinations of the above procedures were applied.

Results

It was demonstrated in a previous study that basic dyes, if applied after pretreatment with iron alum, stain acid mucopolysaccharides in an elective manner. To obtain such staining it is not necessary to acidify the aqueous solutions of the dyes.

It was further demonstrated that iron hydroxide ions, responsible for elective staining, were bound to the acid-mucopolysaccharide-containing structures and to the non-staining tissue components alike.

The polyanions of acid mucopolysaccharides do not react with basic dyes when blocked by methylation or treatment with CPB. The reactions are likewise negative if the procedure is followed by iron alum treatment. However, all structures give positive iron-sulphide reaction if blocked tissues are treated with iron alum and subsequently with ammoniumsulphide or potassium ferrocyanide. In the blocked cartilaginous ground substance both reactions are less marked than in the non-blocked controls (Figs 1, 2).

After hyaluronidase digestion, the cartilaginous substance failed to bind basic dyes, while its iron-binding capacity, though less pronounced, remained demonstrable.

Iron-alum pretreatment following sulphation failed to yield elective staining. The iron-sulphide reaction appeared, however, intensive and homogeneous if the sulphated and iron-alum pretreated sections were immersed in ammonium sulphide.

Staining with basic dyes and subsequent treatment with iron alum for 10 to 15 minutes resulted in elective staining of the acid mucopolysaccharides (Fig. 3). All other tissue components were discoloured by iron alum.

Previous trypsin digestion did not weaken the staining reaction of acid mucopolysaccharides, but did weaken that of the surrounding tissues. In

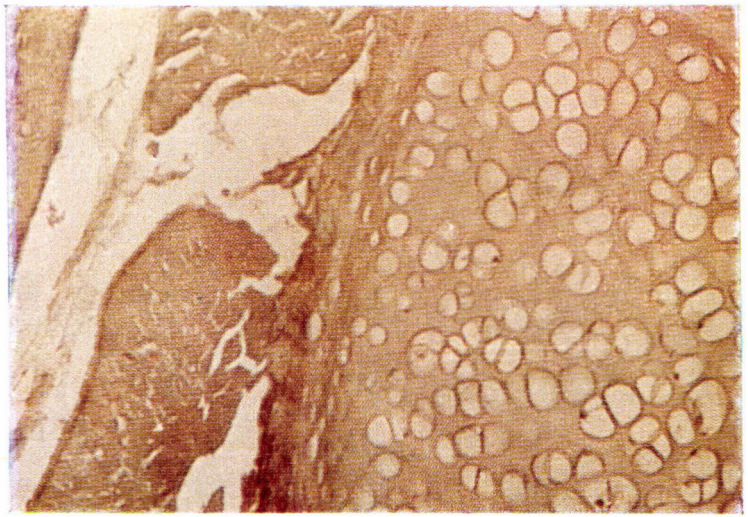

Fig. 1. Section of costal cartilage of rat. Iron alum and ammonium-sulphide. ×200

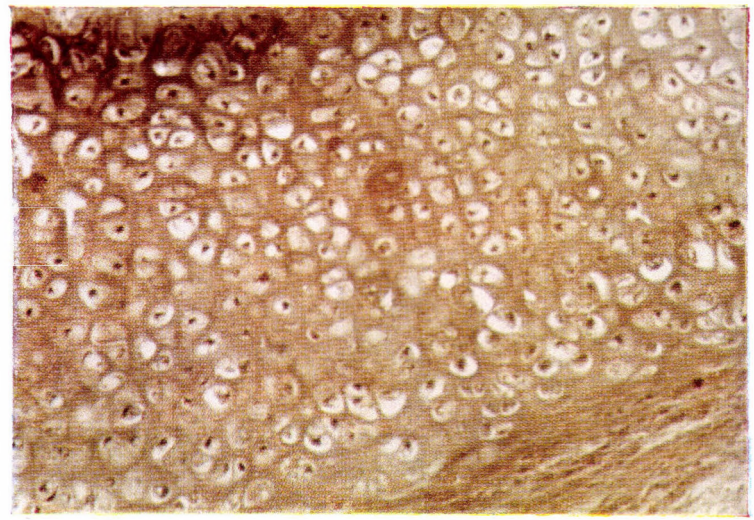

Fig. 2. Section of costal cartilage of rat. Methylation followed by iron-alum—ammoniumsulphide reaction. ×250

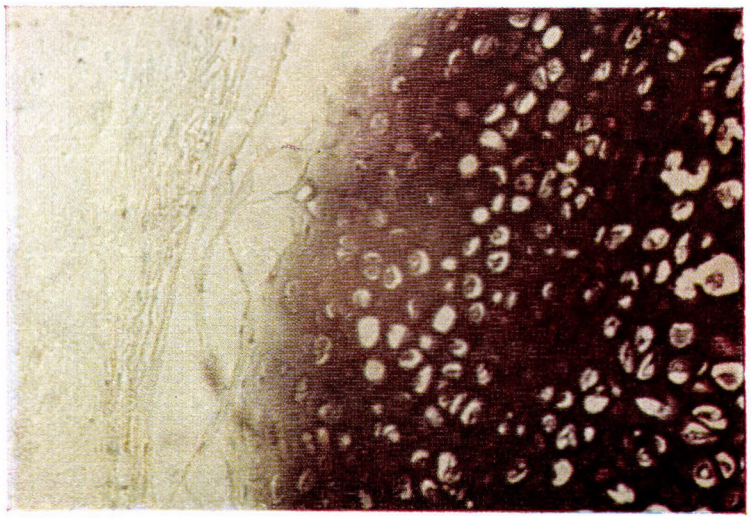

Fig. 3. Section of costal cartilage of rat. Staining with Azure I followed by treatment with iron alum. ×250

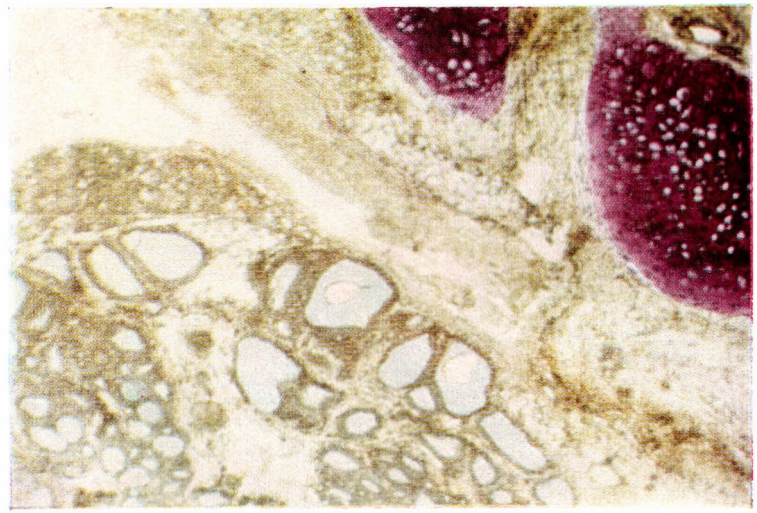

Fig. 4. Section of laryngeal cartilage of rat. Staining of control preparation with Azure I. ×100

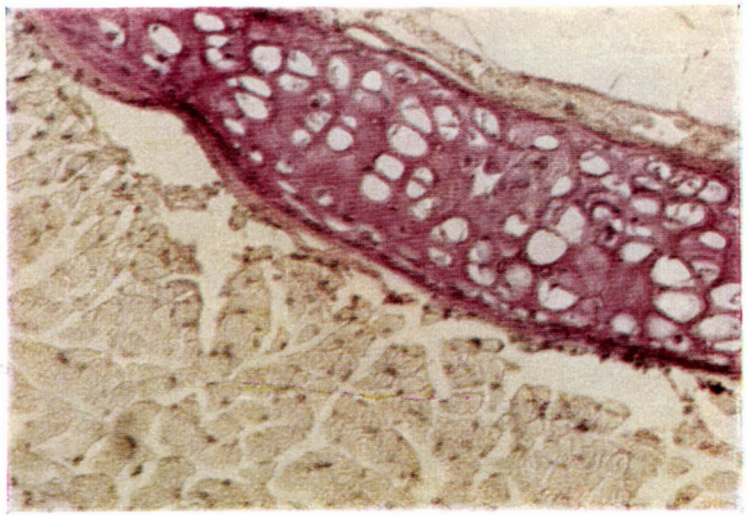

Fig. 5. Section of laryngeal cartilage of rat. Staining with Azure I after digestion with papain. ×200

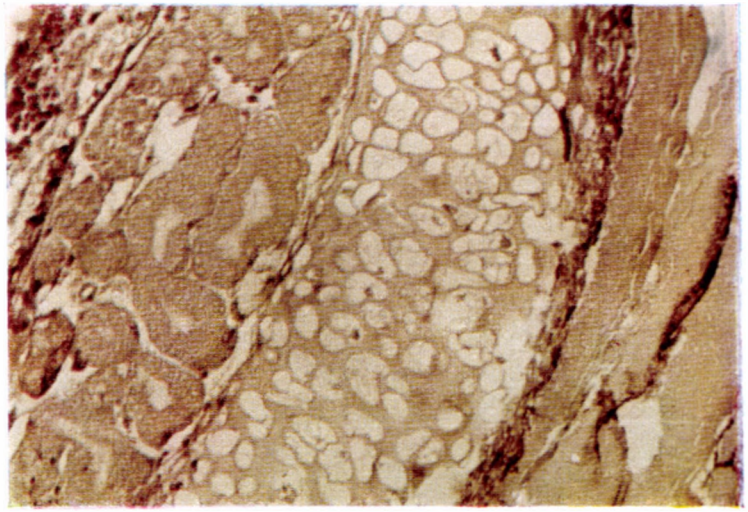

Fig. 6. Section of rat larynx. Result of iron-sulphide reaction after digestion with papain. ×250

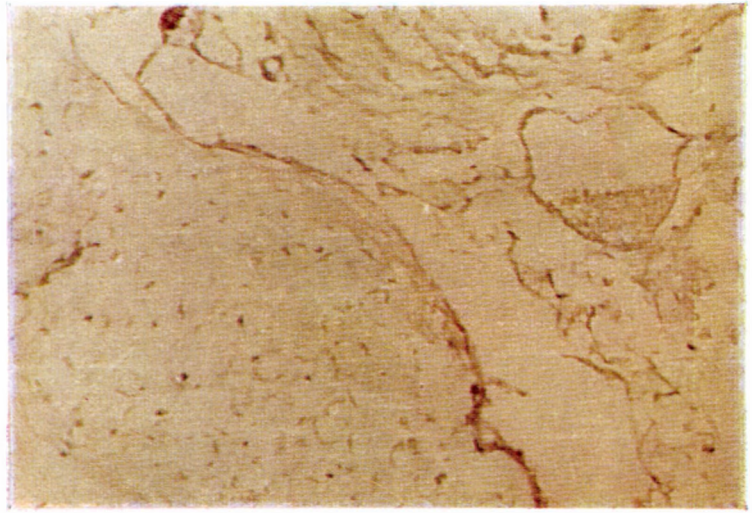

Fig. 7. Section of rat larynx. Iron-sulphide reaction following methylation and papain digestion. ×200

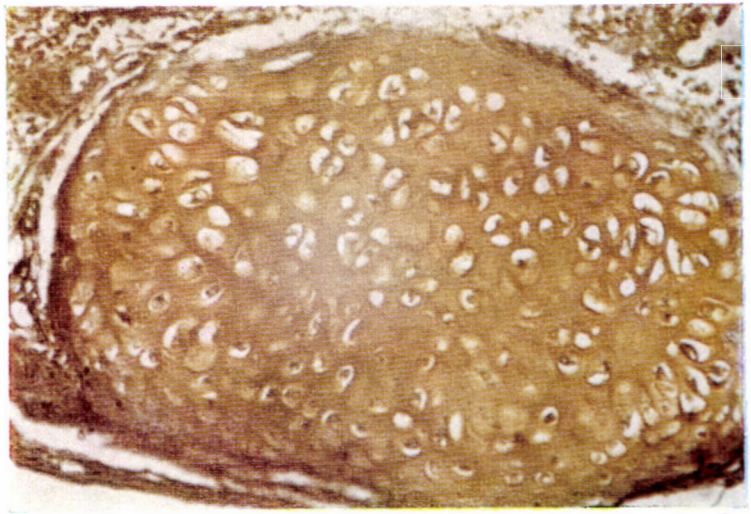

Fig. 8. Section of rat larynx. Result of staining with acriflavine and iron-sulphide reaction performed after pretreatment with iron alum

trypsin-digested and iron-alum-treated tissues the iron binding remained unchanged in the cartilage ground substance while it became weaker elsewhere.

Papain digestion, as short as 30 minutes already diminished the intensity of staining; digestion of 4 hours resulted in a conspicuous reduction of the stain-binding capacity (Figs 4, 5), but the fresh, wet preparations so treated still showed metachromasia. It is worthy to note that digestion with papain did not significantly affect the staining of mast cells.

Iron-sulphide reaction performed after papain treatment diminished the iron-binding capacity of the cartilaginous ground substance quite considerably, whereas the intensity of iron-sulphide reaction in the adjacent tissues was not significantly reduced (Fig. 6).

Methylation of the sections after papain treatment resulted in the total loss of both the dye- and iron-binding power by the structures containing acid mucopolysaccharides (Fig. 7).

In another series of experiments, the elective staining of acid mucopolysaccharides was combined with the iron-sulphide reaction. Preparations were pretreated with iron alum, electively stained with acriflavine, a dye giving sharp contrast, and then placed in a solution of ammonium sulphide. The tissues containing acid mucopolysaccharides assumed a brownish hue, while the other tissues became dark brown. Substitution of ferric chloride for iron alum gave the same result (Fig. 8).

If iron-sulphide reaction preceded the staining with acriflavine the most part of the iron-sulphide was expelled from the surface of the tissues. Results are listed in Table 1.

Table 1

Colour reactions of structures containing acid mucopolysaccharides

Pretreatment	Reactions	
	Iron sulphide	Azure I
Iron alum	+++	+++
Methylation + iron alum	+	−
CPB + iron alum	+	−
Hyaluronidase + iron alum	+	−
Trypsin + iron alum	++	++
Papain + iron alum	+	+
Papain + methylation	−	−
Iron alum + acriflavine	+	0
Sulphation + iron alum	++++	++++

Discussion

The fundamental phenomenon in the chemical mechanism of elective acid mucopolysaccharide-reaction is the binding of ferric ions. Recently WIGGLESWORTH [8] has studied in detail the binding of metal ions (especially of iron ions) by tissue structures. He treated sections of differently fixed tissues with iron alum and demonstrated the tissue-bound iron by treating with ammonium sulphide. Among the components of tissues nucleic acids and proteins were found to bind the most iron. The phosphate groups of nucleic acids and the carboxyl groups of proteins proved to be directly responsible for the binding of iron; these groups form, according to WIGGLESWORTH, a non-ionizing complex with the iron. The intensity of the iron-sulphide reaction (i.e., the staining) is proportional to the acidity of the reacting structures i.e., to the relative quantity of their iron-binding radical. WIGGLESWORTH's investigations have moreover proved that ferrous ions are less strongly bound by the tissues than are ferric ions.

For binding of basic dyes, the anions of the tissues are responsible. Considering that ferric ions form strong non-ionizing bonds with these anions, it will be understood why the pre-existent anion-iron complexes inhibit the binding of basic dyes, i.e., stainings, in the case of containing no acid mucopolysaccharides. The question arises here as to why structures containing acid mucopolysaccharides, which also bind iron, do give positive reaction with basic dyes. It is obvious that their iron-binding capacity is also based on their anion contents. However, unlike other tissue elements, in acid mucopolysaccharides sulphate ions are predominant. It seems therefore reasonable to suppose that the sulphate radicals are responsible for this elective staining.

The bond between sulphate radicals and iron ions is evidently less firm than that between iron ions and other anions, a phenomenon which makes a competitive antagonism between iron ions and basic dye-molecules possible. Consequently the molecules of the dye displace the ions of iron from the sulphate bonds. It can be presumed that the carboxyl groups of the acid mucopolysaccharide-containing structures are not involved in the binding of dyes.

Our investigations confirm this supposition. Elective staining could be prevented by previous sulphation because in this case sulphate-iron binding predominates also in the tissues containing no acid mucopolysaccharides.

That the bonds existing between ferric ions and sulphates are different from those between iron ions and phosphates and carboxyls was demonstrated in the experiments in which the order of treatments was reversed, i.e., staining with basic dye preceded the iron alum treatment. As a result of iron alum treatment, the staining of the tissues containing no acid mucopolysaccharides gradually disappeared, while that of the acid mucopolysaccharide-containing tissues remained unchanged. We advance the following interpretation of this

phenomenon. In view of the nature of the competitive antagonism existing between the molecules of basic dyes and ferric ions it is obvious that ferric ions are not able to displace the dye molecules bound by the acid mucopolysaccharides. The strong affinity of ferric ions to the phosphate and carboxyl groups, and the nature of the complexes so formed, make at the same time clear why dye molecules are expelled from the other tissues by the ferric ions.

The next question we were faced with was whether the molecules of basic dyes displace the iron ions completely from the acid mucopolysaccharide-containing structures. We have seen that the ammonium-sulphide reaction, performed after the elective staining of acid mucopolysaccharides, revealed the presence of iron ions in the cartilaginous ground substance. Since, however, the colour of the dye (e.g., acriflavine) interfered with the evaluation of the sulphide reaction, in later experiments the sulphate radicals were blocked with CPB, which is a colourless substance. The iron-sulphide reaction so obtained was less intensive but well perceptible. As the iron-sulphide reaction was positive after methylation and after digestion with hyaluronidase as well, it seems to be obvious that the protein component of the acid mucopolysaccharide complex, too, binds iron.

It is known from the literature that in chondro- and mucoprotein complexes the proportion of the protein component is considerably large [2]. Analysis of the amino acids contained in these proteins shows a relatively high percentage of dicarbonic acids (e.g., aspartic acid, glutamic acid). It has already been noted that carboxyl radicals are important factors for the stable binding of iron ions. That the protein component of chondro-mucoproteins cannot be removed by treatment with trypsin is clear from the literature and has been confirm ed by our experiments. Since, according to literary data, this kind of protein is sensitive to digestion with papain, we employed this procedure. The capacity of chondro-mucoproteins to bind iron was found to have considerably diminished in the papain-digested sections. As a result of methylation the iron-binding capacity of the papain-digested chondro-mucoproteins approximately disappeared, indicating that the iron was bound to anions of the acid mu copolysaccharides.

The iron-binding capacity of collagen proteins is, according to WIGGLESWORTH's findings, generally modest. Therefore, the iron-binding property of this component of the cartilaginous ground substance may be neglected.

It is worthy to note that, after papain digestion, the cartilage reacted with basic dyes less intensely than the control preparations, a phenomenon in respect of which we can offer speculative interpretations only. It is, for instance, possible that in the reaction also part of the acid mucopolysaccharides is decomposed by papain. Again, it is not impossible that the protein component of the intact chondro-mucoprotein complex, too, plays some role in the binding of dye molecules.

The heparin-protein complex of mast cells seems to behave differently from the chondro-mucoprotein, a phenomenon which requires further investigation.

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METHODE ZUM ELEKTIVEN NACHWEIS SAURER MUCOPOLYSACCHARIDE MIT EISENALAUN. II

Der chemische Mechanismus einer für den elektiven Nachweis saurer Mucopolysaccharide erarbeiteten Eisenalaun-Färbungsmethode wird untersucht. Die elektive Färbung beruht teils auf der blockierenden Wirkung der Eisenionen über die stabile Bindung der Phosphat- und Carboxylgruppen, teils auf der losen Bindung, die zwischen den Eisenionen und den Sulfatradikalen zustande kommt, und nach dem Prinzip des kompetitiven Antagonismus die Bindung der basischen Farbstoffmoleküle an die sauren Mucopolysaccharide ermöglicht. Der Proteinkomponent des Chondro-Mucoproteins hält die Eisenionen gleichfalls in fester Bindung und spielt vermutlich eine Rolle im Mechanismus der Farbstoffbindung des intakten Chondro-Mucoproteins.

МЕТОД ДЛЯ ИЗБИРАТЕЛЬНОГО ВЫЯВЛЕНИЯ КИСЛЫХ МУКОПОЛИСАХАРИДОВ ЖЕЛЕЗНЫМИ КВАСЦАМИ. II.

Изучался химический механизм метода для избирательного выявления кислых мукополисахаридов с помощью железных квасцов. Установлено, что избирательное окрашивание осуществляется отчасти в результате блокирующего действия ионов железа путем прочного связывания фосфатных и карбоксильных групп, а отчасти в результате непрочной связи, возникающей между ионами железа и сульфатными радикалами, обеспечивающей-согласно принципу компетитивного антагонизма-присоединение молекул основного красителя к кислым мукополисахаридам. Белковый компонент хондро-мукопротеина также связывает в прочной форме ионы железа и, предположительно, играет роль в механизме связывания красителя невредимым хондро-мукопротеином.

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$^{14}CO_2$ ASSIMILATION OF NORMAL AND CHLOROPLAST MUTANT LEAVES AT DIFFERENT LIGHT INTENSITIES

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Synopsis

$^{14}CO_2$ fixation and ^{14}C metabolism were studied in normal leaves and in mutants accumulating ζ-carotene and lycopene, respectively, at various levels of chloroplast differentiation. The intensity of photosynthesis was characterized by the total ^{14}C incorporation. In normal leaves ^{14}C incorporation is highly increased by illumination, while the mutants accumulating ζ-carotene are capable of dark fixation only. In lycopenic leaves the ^{14}C incorporation culminates at 1,000 lux light intensity but considerably decreases at higher light intensity. In normal and lycopenic leaves a linear relation exists between $^{14}CO_2$ fixation and the changes of chlorophyll content. In ζ-carotenic leaves the increase of chlorophyll content observable at 100 lux light intensity is not accompanied by an increased $^{14}CO_2$ fixation. Separation of the alcohol-soluble fraction into ionic and non-ionic fractions revealed that in mutant leaves the ^{14}C incorporation in the non-ionic fraction is hardly or not at all stimulated by increased illumination, while in normal leaves the labelling of the non-ionic fraction becomes more and more prevalent. The specific labelling of the protein fraction demonstrable whith the Folin reagent in normal leaves exceeds that of mutant leaves already at 5 lux light intensity, and this difference is growing with increasing of light. Comparing the specific labelling of free amino acid and protein fractions, it turned out that in normal leaves, contrary to mutant ones, the synthesis of new chloroplast protein was induced by light.

Introduction

The functionable chloroplasts of higher plants develop in the course of a gradual differentiation [8, 31]. Out of the internal and external conditions of differentiation the inner, genetic ones are known only in part, merely their phenotypic effects are observed [21, 22, 34].

By systematic changing the external conditions first of all by changing the light intensity may produce structurally different chloroplast, well observable by electronmicroscope [26].

The irregularities of genetic factors may modify the process of differentiation and as a consequence, the plastid may become sensitive to external effects [5, 18, 21, 33].

The plastids blocked at certain stages of differentiation probably differ also in photosynthetic activity. The question is whether the various chloroplast-mutant plants show differences only in the intensity of CO_2 incorporation or also in the metabolisation of CO_2. We have every reason to assume the latter, because the disorder manifesting itself in the plastid structure may be closely connected to the synthesis of enzyme-protein [16, 29].

It is well know that the incorporation of CO_2 by plants depends on two fundamental mechanisms: the dark fixation and the activity of light-stimulated cycles.

The light-independent incorporation of CO_2 is promoted chiefly by the activity of the phosphoenolpyruvate carboxylase enzyme [19, 28], the possibility of the path leading over the keto acids should also be taken into consideration [7].

In light the greatest proportion of CO_2 becomes incorporated according to the known mechanism of the Calvin cycle [6] but at low concentrations of CO_2 the glycolic-acid cycle plays an important role in the fixation [17, 32, 35].

Our previous investigations have revealed that the $^{14}CO_2$ assimilation of extremely light sensitive chloroplast mutant maize plants differs from that of normal plants [13]. The quantitative and qualitative analysis of the incorporated $^{14}CO_2$ has shown that the photosynthetic activity of mutant leaves remains considerably below that of normal plants, and the metabolization of CO_2 is chiefly characterized by the path of dark fixation [15]. In the present paper those data are summarized which were obtained by investigating the connection between chloroplast differentiation and the labelling of some main components.

Material and method

Young seedlings of normal, and two chloroplast mutant maize strains were used in our experiments. One of the mutants is characterized by accumulating ζ-carotene instead of β-carotene. For the second mutant it is typical that the inhibition of carotene biosynthesis in it appears behind the lycopene [10].

At low light intensities (10 to 30 lux) the mutant leaves are able to synthesize chlorophyll, while at intensive light their pigments break down quickly [9, 11].

The grains were germinated in the dark at 28°C, and the seven-day old seedlings were illuminated with different light intensities (5, 100, 1,000 and 10,000 lux). Subsequently the excised leaves were placed in the $^{14}CO_2$ atmosphere, in which the CO_2 concentration was 0.6 volume per cent and the specific activity of ^{14}C to 31 μC per ml.

From a mixture of $Ba^{12}CO_3$ and $Ba^{14}CO_3$ the CO_2 was liberated with 1 N $HClO_4$ in the presence of methylorange indication. In the closed system the air was driven by a membrane pump. After two-hour exposition the CO_2 remaining in the system was fixed with 30% KOH and afterwards the leaves were ground with 90% ethanol. To obtain extracts, various solvents were added to leaf suspension and the fractions were separated by centrifugation.

Alcohol-soluble fraction. The leaf homogenizate was successively extracted with ethanol of 96, 70, 50 and 20% concentration, lipids were removed and the extracts were united.

Lipid-soluble fraction (pigments). From the 96% alcoholic extract the pigments were transferred — by adding petroleum ether and a little water to the extract — into petroleum ether.

Trichloroacetic-acid fraction. From the precipitate remaining after alcoholic extraction the nucleotids were extracted by 5% trichloroacetic acid. The insoluble precipitate was used for preparing further purified fraction.

Starch. The trichloroacetic-acid fraction was washed several times with water and digested for 24 hours with amylase.

Proteins were extracted with 1 N NaOH at 80°C. The residue (polysaccharides) did not show considerable ^{14}C activity.

Each fraction was filled up to a known volume and the ^{14}C content was determined by a GM tube of 1 mg/cm^2 window thickness achieving thus an efficiency of 10%.

The results given in the Tables and Figures are the averages of three parallel measuring series.

Results and discussion

Incorporation of total $^{14}CO_2$

The intensity of CO_2 incorporation was measured by establishing the total ^{14}C incorporated in the leaves (Fig. 1).

At 5 lux light intensity, which may practically be considered as darkness, the total ^{14}C content of normal and mutant leaves is the same. This proves that

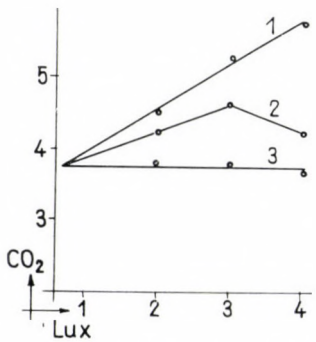

Fig. 1. CO_2 incorporation in normal (1), lycopenic (2) and ζ-carotenic (3) leaves illuminated with different light intensities for 12 hours. Ordinate: log μ mole CO_2/g dry weight. Abscissa: log lux

the mutation does not affect the light-independent incorporation mechanism of CO_2 (dark fixation).

With increasing light intensity, considerable differences are manifest in the CO_2 assimilation of the examined strains.

The relation between photosynthesis and light intensity permits some generalization. The quantity of $^{14}CO_2$ incorporated in the leaves can be looked upon as the sum of two processes: of dark fixation and photofixation.

The light-induced surplus of incorporated CO_2 quantity as a function of light intensity can be expressed by the following equation:

$$y - y_0 = a \cdot x^n \text{ or its logarithmic form:}$$

$$\log (y - y_0) = \log a + n \cdot \log \times$$

where

y_0 = experimental value of dark fixation:
5,630 μ mole CO_2/g dry weight;

y = the actual value of photofixation;

a = the CO_2 quantity incorporated at the light intensity of 1 lux;

n = a constant characterizing the photosynthetic apparatus of the plant;

x = the intensity of illumination

"a" and "n" can be established graphically.

For normal plants the value of the "n" constant, calculated graphically, was 0.635.

For lycopenic leaves on the ascending part of the curve: $0 < n < 0.635$. It is supposed that photofixation of $^{14}CO_2$ depends on two processes: light increases photofixation, but may cause a gradual disorganization of plastids. In normal plants the latter process does not occur. In lycopenic leaves the obtained maximum curve reflects the difference of both processes. For ζ-carotenic leaves "n" = 0 and "y" = constant. This indicates that under the given experimental conditions this mutant is unable to fix CO_2 photosynthetically, only dark fixation occurs in it. The data show that in normal leaves the increasing CO_2 assimilation due to higher light intensity is connected with the differentiation of chloroplasts, the organization of the latter being also a light-dependent process [4]. Thus in lycopenic and ζ-carotenic leaves the photosynthetic apparatus is obviously affected by the mutation.

The ^{14}C activity of the alcohol-soluble fractions and its distribution in their main components

Eighty to 85% of the incorporated ^{14}C was found — independently of light intensity — in the alcohol-soluble fraction. This fraction was separated to cationic, anionic and neutral fractions on ion-exchange resin [27]. Depending on light intensity, distribution of the radioactivity between cationic, anionic and non-ionic compounds reflect a connection between the organization stage of plastids and the CO_2 metabolism.

The activity of the alcohol-soluble fraction and the percentage distribution of ^{14}C activity in the separated components of the examined three strains are presented in Figs 2a, 2b and 2c.

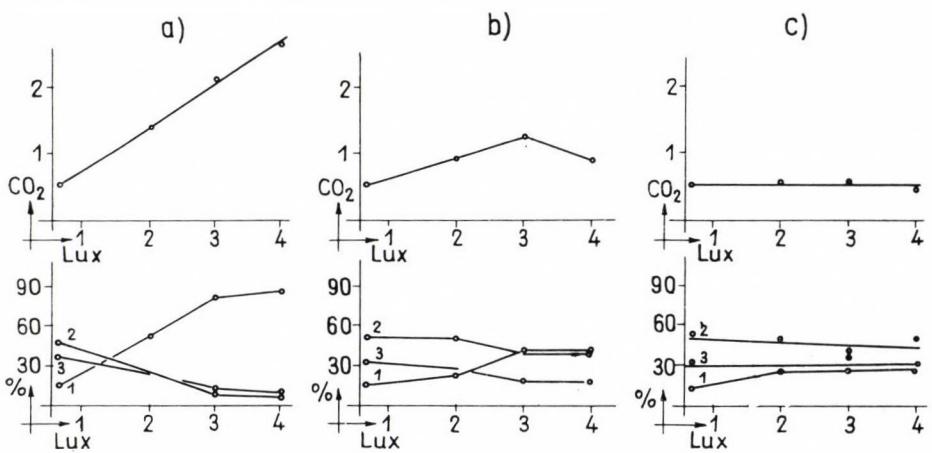

Fig. 2. ^{14}C incorporation in the alcohol-soluble fractions of normal (a), lycopenic (b) and ζ-carotenic (c) leaves illuminated at different light intensities, and the percentage distribution of ^{14}C the subfractions. Upper part: ordinate: log μ mole CO_2/g dry weight. Abscissa: log lux. Lower part: 1 = sugar; 2 = amino acid; 3 = organic acid. Abscissa: log lux.

The total activity of the alcohol-soluble fraction should agree with the total incorporated $^{14}CO_2$.

As to ^{14}C-activity in the various fractions of normal leaves, results consistent with literary data were obtained [1]. A 5 lux light intensity the activity of keto acids and amino acids is considerable, while with increasing light intensity the activity of carbohydrates comes into prominence.

In a similar presentation the percentage distribution of components forming the alcohol-soluble fraction in lycopenic leaves shows a somewhat different picture. Due to increased light intensity, the activity of carbohydrates is low and reaches the value of the ionic components at an illumination as high as 1,000 lux.

In ζ-carotenic leaves the greatest part of ^{14}C incorporated in the alcohol-soluble fractions belongs to amino acids. The activity of sugar remains in every case below that of ionic components.

The data reveal that under the given experimental conditions the ζ-carotenic leaves are not able to incorporate CO_2 in carbohydrates. According to the labelling of free carbohydrates the photosynthetic activity of lycopenic leaves seems to be weaker than that of the normal leaves; this manifests itself also in the total CO_2 fixation.

The most conspicuous steps in chloroplast differentiation are the formation of photosynthetic pigments, the increase of plastid proteins and the organization of the structure [8].

Change of pigment content at different light intensity

If the normal and mutant leaves are illuminated with light of various intensity, great differences occur also in the chlorophyll content. The petroleum-ether extract was transferred into 80% acetone, and from the extinctions measured at the absorption maxima (644 and 663 mμ) of chlorophylls the absolute chlorophyll content (in mg per litre) was calculated according to the method of NYBOM [25] by the aid of the following connection:

$$\text{chl-a:} \quad 9.784 \cdot D_{663} - 0.990 \cdot D_{644} \quad \text{mg/litre}$$
$$\text{chl-b:} \quad 21.426 \cdot D_{664} - 4.640 \cdot D_{663}$$

The data are presented in Table 1.

Increasing the light intensity the chlorophyll content of normal plants tended towards a saturation limit value and was not damaged under conditions of the experiments. With the increasing of light an initial increase in chlorophyll content could be observed also in mutant leaves, but a well-defined decrease occurred in them if treated with light intensities exceeding 100 and 1,000 lux, respectively.

Table 1

The chlorophyll content (mg chl-a + chl-b/g dry weight) of normal and mutant leaves previously illuminated with different light intensities for 12 hours

Material	Light intensity (lux)			
	5	100	1000	10 000
normal	1.1	26.7	39.5	45.8
lycopenic	0.5	6.7	10.4	5.8
ζ-carotenic	0.6	1.4	0.2	0.2

The pigment content of leaves is governed by two light-dependent processes: the pigment synthesis and — in case of strong illumination — the photodestruction [24].

FALUDI and co-workers [12] have proved that in mutant leaves examined in the present work chlorophyll is formed at low light intensities, but it becomes almost entirely broken down if strong illumination is applied. It is difficult to say, which of the aforementioned process is more decisive for the actual chlorophyll content.

The binding of formed chlorophyll molecules to protein is considered to be an important factor. If the formation of protein-pigment complex is inhibited by the abnormality of the protein partner, a rapid destruction of the pigment may take place [14, 21, 23]. The relatively low pigment content of the examined mutant plants is possibly caused by a similar reason.

The ^{14}C labelling of protein

In the course of experiments the quantity and ^{14}C activity of proteins soluble in 1 *N* NaOH were measured. The concentration of protein was established from its alkaline solution, measuring the extinction with Folin reagent [20] at 750 mμ and using a "Spectromom 360" spectrophotometer.

The specific activity of proteins is shown by Fig. 3.

In mutant leaves the specific activity of proteins shows a maximum value at different light intensities; in normal leaves the activity increases with intensified illumination.

It is known that the protein synthesis of plant cells may come about independent of light, but in photosynthetically active tissues the protein synthesis — measured on the basis of the specific activity of plastid protein — has a primary importance [30].

In case of constant protein content, a change (increase or decrease) of the specific activity of the protein fraction indicates a new qualitative step in protein synthesis.

Our previous investigations have proved that in the course of photosynthesis new amino acids are synthesized, which however are hardly detectable among the products of dark fixation [15].

As to the quotients of specific activites of free amino acids and proteins (Table 2) it was found that in normal leaves the increase of light intensity diminishes the ratio, while in the ζ-carotenic mutant the quotients are nearly

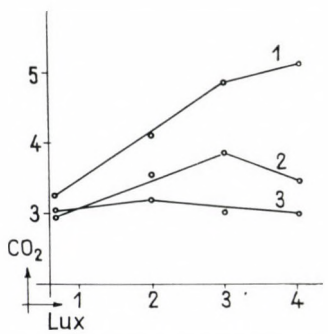

Fig. 3. Specific activity of proteins at different light intensities in normal (1), lycopenic (2) and ζ-carotenic (3) leaves. Ordinate: log μ mole $^{14}CO_2$/mg protein. Abscissa: log lux

identical at different light intensities. In lycopenic leaves till 100 lux a considerable decrease occurred, but at higher light intensities the same values were found as in ζ-carotenic leaves.

Table 2

Rations of the specific activities of the free amino acid fraction to that of the protein fraction at different light intensities in normal and mutant leaves

Material	Light intensity (lux)			
	5	100	1000	10 000
normal	4.2	2.3	0.8	1.0
lycopenic	8.7	2.3	4.8	3.8
ζ-carotenic	6.3	5.7	5.2	6.5

The specific activity of leaf proteins revealed that in normal leaves under the influence of illumination a new protein synthesis, closely connected with chloroplast differentiation, was going on. As to the total protein content there was no difference among the mutants, neither an increase of total protein was caused by intensified light. In accordance with the opinion of other authors [4] we suppose that in forming of plastid proteins also cytoplasmic proteins participate through increasing the pool of free amino acids.

Keeping in mind the experimental result, the synthesis of structural proteins in chloroplasts may follow several alternative ways in normal and mutant leaves (Table 3).

Table 3

The connection of specific labelling of free amino acids and proteins with the synthesis of chloroplast proteins

Specific activity of free amino acid	Type of protein synthesis	Specific activity of protein	Spec. act. of amino acid / Spec. act. of protein
constant "1"	a	constant	constant
	b	increases, decreases	increases, decreases
changes "2"	a	increases, decreases	constant
	b	increases, decreases	increases, decreases

In normal leaves the "2b" path may prevail; this means that in the final phase of plastid differentiation a synthesis of qualitatively new proteins is induced by light.

The data of the ζ-carotenic leaves suggest that in this case the "1a" path is probable, i.e. in this mutant the light does not induce the synthesis of plastid-proteins which are important constituents of the functionable apparatus.

From the data obtained with lycopenic leaves it may be concluded that at low light intensities (50, 100, 1,000 lux) the "2b" path, while at higher light intensity (10,000 lux) the "1a" path determines the specific protein synthesis. This mutant demonstrates clearly that the specific protein synthesis of the plastid is considerably governed by the light.

The experiments reported here have not revealed completely the connection between chloroplast abnormity and protein synthesis. Only the tendencies have been established. More detailed analysis of plastid proteins and electron-microscopic examinations are under preparation.

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$^{14}CO_2$-ASSIMILATION NORMALER UND CHLOROPLASTMUTANTER BLÄTTER BEI UNTERSCHIEDLICHEN LICHTINTENSITÄTEN

Es wurden die $^{14}CO_2$-Fixation und der ^{14}C-Metabolismus von normalen sowie von ζ-Karotin bzw. Lykopin anhäufenden mutanten Blättern bei unterschiedlichen Graden der Chloroplast-Differentiation untersucht. Die Intensität der Photosynthese wurde durch die Einverleibung von Gesamt-^{14}C charakterisiert. In normalen Blättern nimmt die ^{14}C-Inkorporation mit steigender Lichtintensität hochgradig zu, die ζ-Karotin anhäufenden mutanten Blätter sind dagegen nur zu Dunkelfixation fähig. In Lykopin synthetisierenden Blättern kulminiert die ^{14}C-Einverleibung bei 1000 lux Lichtintensität, und nimmt dann bei stärkerer Beleuchtung erheblich ab. In normalen und in Lykopin-enthaltenden mutanten Blättern besteht zwischen der ^{14}C-Inkorporation und den Änderungen des Chlorophyllgehalts ein linearer Zusammenhang. In ζ-Karotin-haltigen Blättern erhöht die bei 100 lux Lichtintensität wahrnehmbare Zunahme des Chlorophyllgehalts die Einverleibung von ^{14}C nicht. Die mit Ionenaustausch einhergehende Absonderung der alkohollöslichen Fraktion erbrachte den Beweis, daß in den mutanten Blättern die ^{14}C-Markierung der Ionenfraktion durch Steigerung der Lichtintensität überhaupt nicht oder nur kaum erhöht wird, wogegen in den normalen Blättern immer mehr die Markierung der ionlosen (Zucker-) Fraktion in den Vordergrund tritt. Die durch Folin-Reagent nachweisbare spezifische Markierung der normalen Blätter übertrifft bereits bei 5 lux Lichtintensität die der mutanten Blätter, und der Unterschied wird bei zunehmender Beleuchtung noch größer. Ein Vergleich der spezifischen Markierungen von freier Aminosäure und Proteinfraktionen zeigte, daß durch das Licht in den normalen Blättern — von den mutanten abweichend — die Synthese von qualitativ neuen Chloroplast-Proteinen hervorgerufen wird.

АССИМИЛЯЦИЯ $CO_2{}^{14}$ НОРМАЛЬНЫМИ ЛИСТЬЯМИ И ЛИСТЬЯМИ С МУТАНТНЫМ ХЛОРОПЛАСТОМ ПРИ РАЗЛИЧНЫХ ИНТЕНСИВНОСТЯХ СВЕТА

Поглощение $CO_2{}^{14}$ нормальными листьями и мутантными листьями, накопляющими ζ-каротин или ликопин, и метаболизм C^{14} были изучены при разных степенях дифференциации хлоропластов. Интенсивность фотосинтеза авторы выражают размером инкорпорации общего C^{14} (рис. 1). В нормальных листьях по мере повышения интенсивности света в значительной мере увеличивается поглощение C^{14}. Мутантные листья, накопляющие ζ-каротин, способны лишь к фиксации в темноте. В листьях, синтезирующих ликопин, инкорпорация C^{14} показывает максимум при интенсивности светав 1000 люксов, а при более сильном освещении она в значительной мере снижается. В нормальных и содержащих ликопин мутантных листьях между инкорпорацией C^{14} и изменением содержания хлорофилла наблюдается линейная связь. В листьях, содержащих ζ-каротин, увеличение содержания хлорофилла, наблюдаемое при интенсивности света в 100 люксов, не повышает инкорпорацию C^{14}. Ионообменное разделение растворимой в спирте фракции выявило, что в мутантных листьях повышение интенсивности света не или мало увеличивает мечение неионной фракции изотопом C^{14}, тогда как в нормальных листьях все более получает перевес мечение неионной (сахарной) фракции. Специфическое мечение выявляемых с помощью фолина белковых фракций нормальных листьев уже при интенсивности света в 5 люксов превышает мечение мутационных листьев, и эта разница увеличивается по мере повышения интенсивности света. Сравнение специфического мечения фракций свободных аминокислот и белков показывает, что в нормальных листьях, в отличие от мутантных, свет обусловливает синтез качественно новых хлоропластовых белков.

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STUDIES IN STOMATAL BEHAVIOUR

EFFECTS OF MOVING AND STILL AIR ON THE STOMATA OF SOME PLANTS

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Synopsis

Experiments have been performed to investigate the effects on stomata of moving normal and still air within porometer cups attached to illuminated leaves of *Datura stramonium*, *Pelargonium zonale*, *Duranta plumieri* and *Clerodendron inerme*. Changing from moving to still air brought about a very marked opening response in *Datura* and *Pelargonium*. An opposite closing effect was found in *Duranta* and *Clerodendron*. The present results are not consistent with the hypothesis that the wide opening of stomata in still air is caused by reduction in the carbon dioxide content of the enclosed air, owing to photosynthesis. The possible causes of the discrepancy are discussed.

Introduction

WILLIAMS [7, 8], HEATH and WILLIAMS [5] were the first to observe that strips taken from the area of a leaf enclosed by an unswept porometer cup show much wider stomatal pores than those taken from the rest of the leaf area.

The abnormally wide stomatal opening within porometer cups was shown by HEATH [6] not to be due to pressure of the cup or to temperature differences, nor to high humidity of the enclosed air. He came to the conclusion that such wide opening of stomata was caused by reduction, owing to photosynthesis, in the carbon dioxide content of the enclosed air below the normal 0.03%.

HAFEZ [2] obtained consistent results showing that cherry laurel stomata opened as much as five times wider in still than in moving dry air. In a later investigation, HAFEZ [3] found that *Eupatorium* stomata tended to close to a greater extent in non-moving than in moving air. In moving and still air, no appreciable difference in the pore areas of *Mentha* stomata could be detected. HAFEZ concluded that his results appear inconsistent with the carbon dioxide hypothesis previously suggested to explain the abnormally wide opening of stomata of some plants within unswept porometer cups.

The main purpose of the present investigation [9] is (1) to find out whether such abnormally wide stomatal opening within porometer cups occurs in plants other than those investigated by earlier workers, and (2) to test the hypothesis [1, 6] that such wide opening is due to reduction by photosynthesis of the carbon dioxide content of the enclosed air below the normal 0.03%. This

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paper describes the results of using repeated cycles of moving and still air on the stomata of some plants.

Material and method

Leaves of *Datura stramonium* LINN., *Pelargonium zonale* L'HERIT., *Duranta plumieri* JACQ., and *Clerodendron inerme* GAERTH. were used in the present investigation.

The design of the porometer cup and the method used in following the changes in stomatal aperture were those adopted by HAFEZ [2]. The cup (Fig. 1) of 7 mm. internal diameter was surrounded by a rubber tube. Immediately behind the tube was inserted a side tube attached to a two-way tap (t_4) which served as an inlet for a steady current of dry air and therefore the air within the cup was not subject to changes in humidity or in oxygen or carbon dioxide tensions. In order to keep the air passing evenly over the leaf surface without dead spaces the inside nozzle of the side tube was drawn out and bent towards the mouth of the cup.

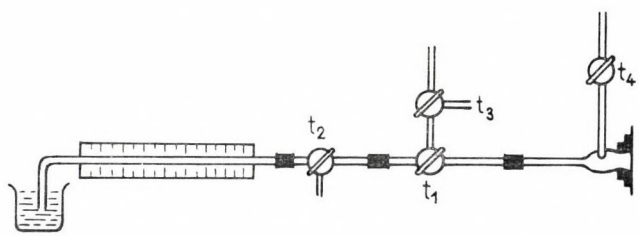

Fig. 1. Porometer; see text for description

Air was drawn through the leaf into the porometer cup by connecting the free end of the latter to a head of water in a narrow-bore horizontal tube with a short vertical arm dipping into a reservoir of cooled boiled water. Thus a constant pressure difference of 7.5 cm of water was obtained throughout the present series of experiments. The rate of flow of the air was measured by the rate of movement of the water meniscus, and this rate was taken as an indication of the degree of opening of the stomata.

Two three-way taps were inserted between the cup and the horizontal capillary tube. One of these (t_2) served to place the capillary tube in communication either with the cup (while a porometer reading was to be taken) or with the exterior (as when the water column in the capillary tube needed to be sucked back to its original position after readings). By means of the other tap (t_1), the porometer could be introduced into the air-sweep circuit between readings. Another three-way tap (t_3) was connected to (t_1) so as to keep the suction pump working while readings were to be taken.

In conducting an experiment, a selected leaf was detached under water and, with its petiole dipping in water, its lower surface was attached to the porometer cup by merely pressing it lightly against the greased edge of the cup. Dry air was circulated through the cup at a rate of 4 litres per hour. The temperature around the leaf was maintained at 23 ± 1 °C.

After a period of time, with the leaf in diffuse daylight, the rates of flow of the water meniscus in the porometer tube were measured at 5-minute intervals. For a state of equilibrium to be reached before taking a reading, twenty seconds were allowed to elapse after placing the column of water in communication with the porometer cup. The time required for the meniscus to move one cm was then determined. The air current was thus interrupted for periods of roughly half a minute while the porometer readings were to be taken. The leaf was next illuminated, in addition to diffuse daylight, by a 1,000-watt lamp with its filament at 120 cm from the leaf surface; the light intensity at the leaf surface being about 1,500 foot-candles. A cooling screen of running water was used to cut off the greater part of the heat.

When the new porometer readings seemed to have attained a reasonably steady value, the air current circulating through the porometer cup was stopped for given periods during which readings were also taken.

Results and discussion

In the following experiments, leaves of the four tested plants were submitted to repeated moving and still air periods during which 5-minute porometer readings were continually recorded. Five experiments were carried out with each plant. The results are presented in Table 1.

Table 1

Half-hourly means of 5-minute porometer readings expressed as percentages of initial rates (just before the first change from moving to still air), shown by the stomata of the four tested plants after half-hour and 1-hour periods of still air following moving air

Experiment No.	Flow rate after		Experiment No.	Flow rate after	
	half-hr. period	1-hour period		half-hr. period	1-hour period
Datura stramonium			*Pelargonium zonale*		
1	150.4	232.7	6	88.0	114.1
2	206.1	295.1	7	101.5	119.3
3	112.4	174.1	8	127.2	157.1
4	150.0	238.8	9	96.7	220.1
5	127.0	178.8	10	94.5	150.3
Totals	745.9	1219.5		507.9	760.9
Means	149.2	243.9		101.6	152.2
% increase	49.2	143.9		1.6	52.2
Duranta plumieri			*Clerodendron inerme*		
11	71.4	69.2	16	87.2	86.1
12	86.5	84.5	17	83.9	74.1
13	80.6	84.2	18	68.8	65.6
14	80.4	77.3	19	77.5	72.5
15	86.5	77.7	20	70.6	64.9
Totals	405.4	392.9		388.0	363.2
Means	81.1	78.6		77.6	72.6
% depression	18.9	21.4		22.4	27.4

Since the results on the action of any one condition on any one plant are generally uniform, to illustrate the action of each condition on each plant only one representative experiment has been chosen and its individual readings, as 100/seconds per 1 cm. length, are plotted against time (Fig. 2). Broken lines indicate that some readings were not taken. Allowance was made for this in calculating half-hourly mean rates.

Table 1 and Fig. 2a—b show clearly that periods of still air following moving air, usually brought about a slight temporary closing movement of the stomata of *Datura* and *Pelargonium*, followed by a gradual and considerable opening movement as shown by the rising flow rate through stomata. When the normal air stream was restored the immediate effect on the flow rate was usually a temporary rise, for about 5 minutes, followed by a gradual significant fall. Similar effects were again obtained under repeated periods of still and moving air.

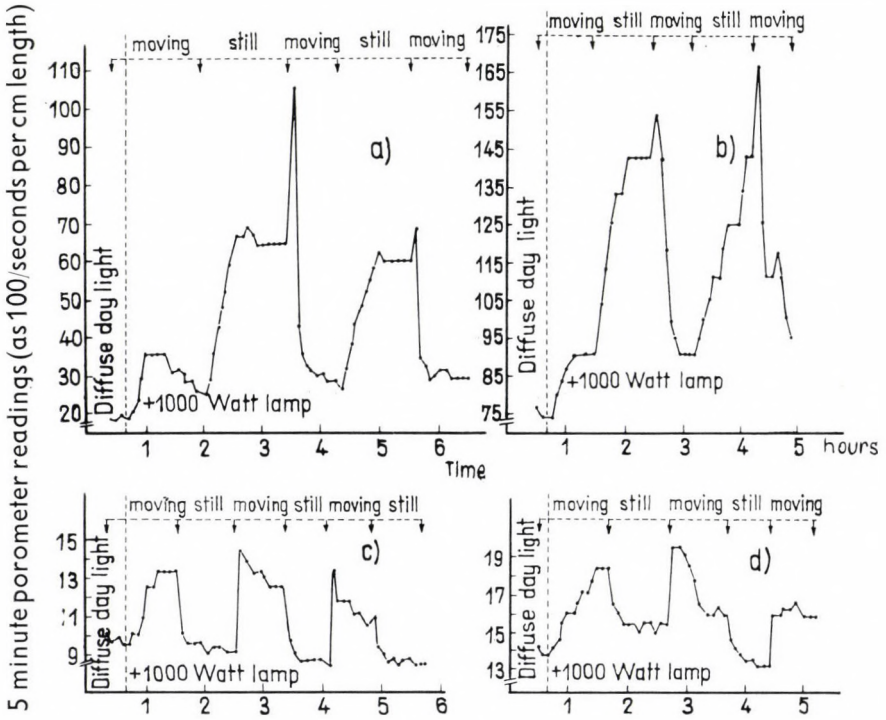

Fig. 2. The effects of moving and still air on the stomata of:
a—*Datura stramonium* (data from experiment 4),
b—*Pelargonium zonale* (data from experiment 8),
c—*Duranta plumieri* (data from experiment 11),
d—*Clerodendron inerme* (data from experiment 16)

With *Duranta* (Table 1 and Fig. 2c) and *Clerodendron* (Table 1 and Fig. 2d), on the other hand, periods of still air brought about opposite effects; the stomata having shown an immediate closing response as indicated by a clear fall of the flow rate through them. This closing response was either maintained or slightly accentuated during the still air period. On restoring air flow the stomata showed an immediate and significant opening movement.

It should be mentioned that, although the stomata of the four test plants showed in many of the experiments a tendency to decrease their pore areas

throughout the period of experiment, yet, such a drift with time was statistically shown not to mask the expected responses to the applied air conditions.

Thus, the results of the present work as well as those of GREGORY and co-workers [1], HEATH [6] and HAFEZ [2, 3] would appear to leave no doubt that plants, in general, respond differently to still air. In the light of an extensive body of results showing that *Pelargonium* and wheat stomata open wider in still than in moving air, HEATH [6] was led to support strongly the view that the abnormally wide opening of stomata within a porometer cup was caused by a reduction, through photosynthesis, in the carbon dioxide of the enclosed air.

Here, again, the four plants of the present investigation responded differently to still air as well as to carbon dioxide-free air [4] under more or less similar experimental conditions. Thus, whereas changing from moving dry to still air brought about a very marked opening response in *Datura* and *Pelargonium*, an opposite effect was found in *Duranta* and *Clerodendron* stomata.

Such discordant responses, reported here and elsewhere, of different plant species to still air would seem not to be in agreement with the carbon dioxide view referred to above. All the evidence for such a view was based on the observation of increased stomatal opening in still air with an illuminated leaf of *Pelargonium* and wheat [1, 6]. Further observations on other plants indicate that such a stomatal response is by no means common, and that stomata may even close more in still than in moving air. In particular may be cited the significant stomatal closure, on changing from moving normal to still air, found in *Eupatorium* [3] as well as in *Duranta* and *Clerodendron* of the present work.

It would seem, then, that the carbon dioxide hypothesis is by no means satisfactory for complete explanation of the abnormally wide opening of stomata in still air. This, of course, does not rule out the possibility of a carbon dioxide effect. But the fact that stomata of different plants respond differently to still air shows that a simple carbon dioxide view does not explain all the facts. Furthermore, the rapidity with which the stomata of many plants open or close on changing from moving normal to still air and *vice versa*, is too great to be entirely due to a reduction, by photosynthesis, of the carbon dioxide content of the enclosed air.

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UNTERSUCHUNG DES VERHALTENS DER STOMATA

Die Wirkung bewegter und unbewegter Luft auf die Spaltöffnungen einiger Pflanzen

Zur Aufklärung der Wirkung von bewegter normaler und unbewegter Luft auf die Spaltöffnungen wurden in Orometerschalen, die an beleuchtete Blätter von *Datura stramonium*, *Pelargonium zonale*, *Duranta plumieri* und *Clerodendron inerme* befestigt waren, Versuche durchgeführt. Der Wechsel von bewegter und unbewegter Luft bewirkte in den *Datura*- und *Pelargonium*-Blättern eine merkliche Öffnungsreaktion. Ein umgekehrter Schließeffekt wurde an den *Duranta*- und *Clerodendron*-Blättern beobachtet. Der Widerspruch zwischen diesen Ergebnissen sowie der Hypothese, wonach das weite Öffnen der Stomata bei unbewegter Luft durch die Photosynthese-bedingte Abnahme des Sauerstoffgehaltes in der abgeschlossenen Luft verursacht ist, wird besprochen.

ИССЛЕДОВАНИЕ ПОВЕДЕНИЯ УСТЬИЦ

на Действие движущегося и неподвижного воздуха на устьица некоторых растений

Проводились опыты по изучению действия нормального движущегося и неподвижного воздуха на устьица в орометрических чашечках, прикрепленных к освещенным листьям растений *Datura stramonium*, *Pelargonium zonale*, *Duranta plumieri*, *Clerodendron inerme*. Смена движущегося воздуха с неподвижным привела к очень выраженной реакции открытия у растений *Datura* и *Pelargonium*. В противоположность этому, у растений *Duranta* и *Clerodendron* была получена реакция закрытия. Обсуждается противоречие между полученными результатами и предположением, согласно которому широкое открытие устьиц при тихом воздухе обуславливается уменьшением углекислоты в закрытом воздухе вследствие фотосинтеза.

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RECENSIONES

Das Versuchstier

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S. Karger, Basel—New York, (1969) pp. 80, DM 24.
(The Laboratory Animal Symposium at the 27th Annual Meeting of the Swiss Microbiological Society, Neuchâtel, 14—15. June, 1968.)

Volume 7 in the series of Bibliotheca Microbiologica includes eight papers, out of which seven were written by experts of the Tierfarm Ag. (Sisseln, Switzerland). This is why the activities, problems and results of this laboratory animal producing institute of very high standard, are treated most thoroughly.

In two Tables of outstanding value R. LOOSLI and H. HURNI give a summary of the complicated relations between breeding methods and hygienic standards usable in laboratory animal breeding. In another paper R. LOOSLI underlines the importance of maintaining animal strains in a genotypically constant state and points to the breeder's difficulties. The next paper by H. HURNI is a literary review (77 references), enriched by some experiences of his own, on the role of environmental factors. H. OHDER discusses microbiological problems of the establishment and maintenance of germ-free and SPF animal units: the unsatisfactory way of food sterilization, the insufficiency of hysterectomy, the role of microbes entering the SPF barrier over and over again, and the way of their control. H. HURNI offers some new ideas on the operation of a germ-free plastic isolator with special regard to hazards arising within the working isolator. MARGIT HAMMERLI and JOHANNA HURNI introduce how to raise gnotobiotic mice and rats on fostermothers; it is noteworthy that they have bred in the isolator guinea pigs, rabbits and cats artificially, i.e., from hand. Useful advice is given by A. ZÜGER concerning the individual hygiene of the personnel of SPF colonies as well as regarding the way how to make the barrier work.

Finally, J. BONNOD, G. DE SAINT-AUBERT and P. FORISSIER spoke about possibilities, conditions and bacteriological control of pathogen-free breeding of mice and rats in industrial size.

L. KÁLLAI (Budapest)

The germ-free animal in research
Ed. by COATES, MARY E. Academic Press, London—New York (1968) pp. 289, 70 s.

"It was about sixty years ago — begins Professor VERZÁR his remembrances in the Preface — that Professor NUTTAL from San Francisco visited my chief, Professor TANGL, in the Laboratory of General Pathology of the University of Budapest . . ." It is a great pleasure to see these Hungarian names and, further, when reading the list of authors, our eyes are caught by the names of H. A. GORDON and T. Z. CSÁKY. Experts of Hungarian birth are, again, among the leading experts of the latest sprout of biological sciences: gnotobiology. The other authors, such as H. BAUER, D. W. VAN BEKKUM, R. FULLER, T. F. KELLOGG, J. R. PLEASANTS, B. S. REDDY, E. SACQUET, J—C. SALOMON, P. C. TREXLER, C. K. WHITEHAIR and B. S. WOSTMAN had also made their names imperishable in this field of research.

In Chapter 1, E. SACQUET discusses equipment, design and management. In his institute, in the French centre for laboratory animal breeding at Gif-sur-Yvette several Hungarian research workers have become acquainted with the germ-free technique. The Shinfield Institute of the University of Reading and the Lobung Laboratory of the University of Notre Dame are

also well-known for the home scientific workers. The following chapters deal with the transport of germ-free animals, their routine microbiological control, production and rearing, as well as their feeding. The other part of the book is devoted to the characteristics of the germ-free animal. H. A. GORDON ventures to answer the question: "Is the germ-free animal normal?" — and he illustrates with several examples that it is not. Yet, "they have shown us, for the first time, what happens when, free of commensals, they are left to their own resources". After a discussion of nutrition and metabolism, the defence mechanism characteristics and the carcinogenesis of axenic animals as well as their characteristics revealed in radiation biology are treated. The book is completed by a considerable bibliography to each chapter, and by an author index.

L. KÁLLAI (Budapest)

FRASER, A. F.: **Reproductive Behaviour in Ungulates**
Academic Press, London—New York (1968), pp. 202, 13 tables, 77 figures, 55 s.

Behavioural research work has become an increasingly widening branch of biology. FRASER's book is the first published in this branch which concentrates on reproductive behaviour. It is worthy of especial note that the species dealt with have (with few exceptions) so far been rather neglected. Former works were mostly concerned with monkeys and rodents, treated only of certain special aspects which did not include problems of reproduction. The animals presented and discussed in this book are four members of the family of Equidae; two Rhinocerotidae; two Suidae; the hippopotamus; two Camelidae; a tragulus (chevrotain); twelve Cervidae; the giraffe, antilocapra; nine bovines (including tragelaphi, taurotragi, bubalis, boves, syncerus, bison); the cephalophus; eleven Hippotraginae (including kobites, hippotragi, oryxes, addax, damaliscus, alcelaphus, connochaetes); seven Antilopinae (including oreotragus, raphicerus, madoque, antilope, aepyceros, litocranius, gazelle); eleven Caprinae (including saiga, oreamnos, rupicapra, ovibos, hemitragus, caprae, ammotragrus, oves).

The work discusses reproductive behaviour in respect of the said species and analyses the effect produced by the endogenous and exogenous factors of reproduction on the consummary action of the animals *via* the cortex, thalamus and hypothalamus. It deals thereafter with tactile, auditory, visual, olfactory, photo-periodic, physical and social environmental influences, and their perception through the receptors, the cortex and the synapses. After a discussion of the endocrine apparatus, the author examines the processes of learning and conditioning, the instinct mechanism as also the seasonal and climatic influences. The next subjects dealt with are oestrus; the behaviour of males prior to mating; reproductive behaviour in respect of mating, conception, parturition; the phenomenon of cannibalism; the animals' behaviour in connection with lactation and the rearing of the offspring; behaviour of the offspring in connection with delactation and puberty. The book concludes with a bibliographical list containing 519 English, French, Japanese, Polish, German, Italian, Russian and Swedish publications.

Cs. ANGHI (Budapest)

GREEN, J.: **The Biology of Estuarine Animals**
Sidgwick and Jackson, London (1968), 21 s.

The book contains 13 chapters covering 402 pages. The author offers detailed descriptions regarding the structural features and dynamics of the deltas and estuaries of rivers, the concentration of salt in (and other chemical properties of) estuarine waters; the author examines further the characteristic properties of the flora and fauna in these waters and specifies in detail the species composing the zooplankton as well as their form of appearance; a detailed analysis is presented of the benthonic macro- and micro-organisms as also of the piscine and avian fauna and the indigenous parasites. The work continues with a discussion regarding food chains and food cycle.

The deltas and estuaries of rivers exhibit quite special features in respect of the brack contained in them as well as in that of their flora and fauna; these features are of both scientific and economic significance. For instance, the presence of a large mass of multidinous nourishing organisms makes the growth of a valuable population of edible fish possible (e.g., Acipenseridae, Percidae, Pleuronectidae, etc.).

The value of the work is enhanced by the fact that the author explains and analyses the osmoregulatory activity of the organisms living in fresh water, brack and sea water; he expatiates upon those species (e.g., sturgeon — *Acipenser ruthenus*, pike perch — *Lucioperca lucioperca*, etc.) which are capable of surviving for some time in all three kinds of water.

The book under review contains 105 instructive illustrations. Zoologists, physiologists, hydrobiologists and experts of piscatology will undoubtedly derive benefit from studying GREEN's work.

B. PÉNZES (Budapest)

Chemotaxonomy and Serotaxonomy

Edited by J. G. HAWKES. Published for the Systematics Association, by Academic Press, London—New York (1968), Special Volume, No. 2. pp. 300, 84 s.

The book deals with proceedings of a symposium held at the Botany Department, Birmingham University, 15—16 September, 1967.

The volume contains the full texts or extended summaries of the papers presented and the Chairmans' summaries. The subject matter was divided into protein and non-protein studies, and one session to serological and immunological work. Invited were papers on animals, plants and micro-organisms.

The first session was devoted to papers on general protein investigations, electrophoresis, etc. Among them a study on the primary structure of fibrinogens from different mammalian species and a haemoglobin study may be mentioned. Fibrinopeptides seem to be specially suitable for taxonomic purposes; the classification of species according to the amino acid sequences of fibrinopeptides agrees in general with the accepted morphological taxonomy.

The second session dealt with serology and immunology. This showed how wide a field this is from the vertebrate soluble proteins to the serological identification of plant pathogenic fungi, and from plant-family level *(Ranunculaceae, Solanaceae)* to infraspecific variability *(Phaseolus* cultivars and hybrids).

Still, we agree with the opinion of one of the authors: "This work should be regarded as a biological task which should draw in and evaluate all available characters, without over-emphasizing the serological data at the expense of the others."

The third session comprised only two papers, both on nucleic acids. Each presented one of the most promising new techniques of chemotaxonomy. As past studies have shown that ribosomal RNA molecules from different sources display differences in size, composition, secondary structure, and mode of biosynthesis, now a sensitive new method is described here involving limited digestion and separation of fragments. The other study demonstrates DNA-hydridization techniques. Hydroxyapatite provides a simple tool for separating single-stranded from double-stranded DNA. Several DNA comparative experiments demonstrate the usefulness of this technique.

The fourth session was concerned with biochemical approaches not connected with protein studies. So the bile salt differences of vertebrate groups and the biochemistry of metabolic pathways in bacteria were dealt with. Two other papers show that secondary plant constituents have an important place in the application of chemistry to higher plant systematics and taxonomy. A survey of flavonoid pigments in the family Gesneriaceae is shown to support the recent classification. Differences in pigment type at the subfamily level indicate a correlation between chemistry and plant geography. The interpretation of chemical data is helped very much by an understanding of the function of particular classes of substances in plants.

In the fifth session the speakers assessed the value of biochemical and serological data in taxonomy. They think the chemical constituents of organism taxonomically valuable, and these have as advantage over classical characters that we can exactly describe them. Patterns of compounds preferably covering a range of biosynthetic origins are much more useful than solitary substances. Concerning microbial taxonomy the experts characterize both genera and species on biochemically-determined properties, while the serological subdivisions are regarded as subspecific serotypes.

Concerning the Chairman's summing-up we learn with interest his scheme about interrelationship of classes of compounds, but we cannot accept his views on *fortuitous* characteristics of chemical properties. Professor SNEATH's (President of the Systematics Association) *Foreword* is much more acceptable saying "Description and analysis must precede synthesis, and today we are still in the first stage. We can already envisage, however, some outlines of the second stage. The future will be exciting . . .".

P. TÉTÉNYI (Budapest)

GARTER, G .S.: **Structure and Habit in Vertebrate Evolution**
(Gen. Editor: R. PHILLIPS DALES). Sidgwick and Jackson, London (1967), pp. 511, 287 illustrations, 63s.

The work under review is the seventh publication in the series of works dealing with the paramount problems of biology. The present volume, describing the evolution of vertebrates, is a continuation of CARTER's earlier works (Animal Evolution; A General Zoology of the Invertebrates; A Hundred Years of Evolution). Evolution is followed in the present work from the gradual transformation of the primitive bottom-dwelling fish into birds living in the air and then into terrest ial vertebrates. The author analyses all structural, constitutional and occasional functional changes connected with the geographical distribution and adaptive radiation of the vertebrates, and, in general, with everything that has enabled vertebrate animals to survive and attain predominance over different regions of the globe or, else, that has led to the destruction of various vertebrate species.

The author discusses in a modern spirit the role played by DARWIN's natural selection in the process of evolution and its connection with genetic mutations (genes and micromutations). He emphasizes the importance of the oecologic study of the various animal populations and points out that evolutionary changes modifying the entire biology, structure, habit and behaviour of the vertebrates do not necessarily indicate direct adaptation to the environments.

Anatomical comparisons between living and extinct vertebrate species form the nucleus of the evolutionary arguments adduced by the author. The work contains moreover certain references to the evolutionary role of biology, oecology, embryology and reproduction.

The author employs the well-proved principle of reconstruction when offering an instructive interpretation regarding the evolutionally important structural and functional changes undergone by the vertebrates: he tries to explain such changes on the evidence provided by the fossilized remains of long extinct species.

It is emphasized by CARTER that the book under review is not complete. Tocomplete his knowledge, the reader should read well-known textbooks, e.g., ROMER: The Vertebrate Body; Vertebrate Paleontology; YOUNG: The Life of Vertebrates; GRASSÉ: Traité de Zoologie; PROSSER and co-workers: Comparative Animal Physiology.

The work is meant for the use of students of zoology in the first line; both its size and its abundant material of illustrations try to satisfy their requirements, and this purpose is well served by the book's lucid style, the well comprehensible definition of concepts and the summary discussion of the fundamental principles of evolution (chapter XX). However, not only university students but all those will derive benefit from reading the book who wish to gain a better insight into the structure, habit and functions of the vertebrates from an evolutionary angle.

F. BICZÓK (Szeged)

Experiments in Microbial Genetics
Ed. CLOWES, R. C. and HAYES, W. Blackwell, Oxford (1968), pp. 244.

There are works — and the book under review is one of them — which are needed and have, therefore, to be written and published. Microbial genetics have considerably helped during the last two decades in transforming our entire biological outlook, and all those who are receiving higher education need become familiar with its fundamental principles. There exist, moreover, still many unsettled problems, and research workers active in this field cannot hope to solve them without being conversant with the methodology of microbial genetics. The work at issue satisfies this double requirement in an exemplary manner.

What the reader is actually offered is the material of the four-week summer courses of practical microbial genetics held in London in the years 1960, 1961, 1962 and 1964. The curriculum of these courses covered the range of experiments which yielded the most important results in the sphere of microbial genetics.

A general technical guide is followed by the description of three experiments concerning mutation. The production of auxotrophic mutants in these experiments is paving the way for subsequent experiments. The classic fluctuation test of LURIA and DELBRÜCK is necessary for creating the basis of theoretical genetics.

Experiments Nos. 4 to 6 deal with the biochemical characteristics of mutants.

Four experiments acquaint the reader with transformation by means of DNA.

Experiment 10 demonstrates the method of DNA — RNA hybridization. Lac operon was employed for the examination of the activity of genes.

Six experiments (a number in accordance with the significance and the further possibilities of the subject) deal with genetics of bacteriophages. Description of the fundamental microbiological technique is followed by discussions regarding the phenomena of the kinetics of phage growth, chemical mutagenesis, phage crossing, the genetic aspects of phage chromosomes, tempered phages and lysogeny.

Experiments 21, 22 and 23 are concerned with the properties of bacteriophages and their genetics.

Experiments 24 and 25 demonstrate the phenomenon of transduction and colicinogeny.

Experiments 28 to 32 present the methodology of conjugation performed with the *E. coli* strain K-12.

Although the work is mainly concerned with bacterial systems, chapters 33 to 37 make *Aspergillus nidulans* accessible to investigators as a test object.

G. Szabó (Debrecen)

Advances in Microbial Physiology, Vol. 2
Ed. by A. H. Rose and J. F. Wilkinson. Academic Press, London (1968)

The editors have chosen five problematic fields of microbiology, in each of which the progress in the last few years has made a survey easier.

J. Lascelles in her paper on "The Bacterial Photosynthetic Apparatus" discusses the chemical composition, function, and development from the cellular membrane system, of chromatophores. The paper introduces the reader into the processes regulating the development of the chlorophyll synthesis and that of the chromatophore-pigment-protein system.

In the chapter "The Plasmids of *Staphylococcus aureus* and their Relation to Other Extrachromosomal Elements in Bacteria" H. Richmond presents the new classification and frequency distribution of plasmids as well as the combinations of markers which are not yet included in the general classification. Based on transduction experiments the patterns of plasmids, the probable modes of their interactions and the attachment-site hypothesis of compatibility are analysed in detail. A probable marker order is presented. The common features shared by the extrachromosomal elements of Gram-negative bacteria and episomes are pointed out.

In the paper "Life cycle of bacterial ribosomes" W. S. Kelley and M. Schaechter review the data elucidating the synthesis of ribosomal RNA and protein and describe the physico-chemical properties of the ribosomal subunits as well as the pathways in which ribosomes of full functional value are developed and re-organized into polysomes and those in which the latter are again decomposed into subunits.

In K. Beran's article "Budding of yeast cells, their scars and ageing" the processes ensuing in the cell wall during budding, the factors influencing these processes and the age distributions within populations are discussed. The data reviewed are mostly based on fluorescent-microscopic studies.

B. E. B. Moseley's paper "The repair of damaged DNA in irradiated bacteria" deals with the characteristic damages due to UV and ionizing radiation. The still unrevealed steps of the photoreactivation and dark repair are discussed.

The photocopies published in the volume are of good quality and convincing. The Figs and Tables are clear. The bibliography is abundant. The tasteful get-up of the book praises the publishers.

G. Hérmán (Budapest)

The Biochemistry of Virus Replication
Proceedings of the Fourth Meeting of the European Biochemical Societies, Oslo, 1967. Ed. by S. G. Laland and L. O. Fröholm. Universitetsforlaget, Oslo (1968), pp. 107, 50s.

This volume contains the six papers presented at the symposium on The Biochemistry of Virus Replication held during the Meeting named above. The symposium was organized by A. P. Nygaard.

The paper of Spiegelman et al. presents an *in vitro* system for replication of the RNA of the Qβ phage of *E. coli*.

Experiments proving the preference of the phage-induced RNA replicase for the homologous template are described. A complex analysis of the replication cycle of the phage RNA has revealed that the first product possesses the properties of the so-called Hofschneider structure,

subsequently Franklin structures appear and this is followed by the appearance of the infectious Qβ-RNA. The cycle lasted approximately 20 minutes. If transfers were carried out at shortening intervals up to the 74th transfer, replicating molecule had eliminated 83% of its original genome, yet, retained its self-duplicating capacity in the *in vitro* system. According to the authors, this is the smallest known self-duplicating entity at present. The great affinity for the RNA replicase and lack of infectivity of these molecules may open up a novel pathway toward a highly specific device for interfering with viral RNA replication.

Two papers deal with the problems of transcription: a paper of ZILLIG and co-workers deals with the physical structure and composition of a DNA-dependent RNA polymerase of *E. coli*. A model for the regulation of the rate of transcription is derived. The paper of HALL and CROUCH presents hybridization experiments which make distinction between early and late RNA molecules present in the T4-infected *E. coli* cells. The transcription of the late genes of the T4 phage was studied in detail.

Three papers discuss the macromolecule synthesis in T-phage-infected *E. coli* cells from different aspects: SHALITIN deals with the regulation of the protein and RNA synthesis, HASELKORN et al. with the synthesis of early and late RNAs; in both studies the T4 phage-*E. coli* system was used. BACHRACH has shown that regulation of the T2 phage development in infected *E.coli* cells operates at the level of transcription.

There is no doubt that the volume presents the description of some of the very important works carried out in the field of virus research lately. All the authors used combinations of very modern methods. As models phage-bacterium systems were applied, but the results and conclusions may be useful in other fields of virology as well.

An understanding of the experiments requires an expertness in both biochemistry and virology.

E. FARKAS (Budapest)

Advances in Enzyme Regulation. Vol. 6
Ed. by GEORGE WEBER, Pergamon Press; Oxford (1968) pp. 517, 140/—.

This volume of Advances in Enzyme Regulation contains the papers presented at the Sixth Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Tissues. The symposium was held in Indianapolis, USA, 1967.

The subjects in the different sessions show a rather great variation as shown by the titles of the sessions.

I. Regulation of Carbohydrate and Lipid Metabolism.
II. Enzyme Regulation in Man.
III. Steroids: Mechanism of Action.
IV. Isozymes, Development; Action of Vitamin K.
V. Activation and Feedback in Enzyme Regulation.
VI. Regulation of Dihydrofolate Reductase.
VII. Cyclic AMP and Enzyme Regulation.
VIII. Regulation in Cancer.
IX. Special Symposium Lectures (The Effect of Ethanol on the Metabolic Activities of the Liver).

Some of the chapters containing logically coherent papers, cover large fields of science while others only details of the concerned fields. The most extensive session was the first dealing, among other subjects, with the energy-dependent control of the tricarboxylic acid cycle by fatty acid oxidation, with the role of carnitine and long-chain acylcarnitine in the regulation of lipogenesis, and with the interrelations between fatty acid oxidation and the control of gluconeogenesis. A brief paper by KOSHLAND on the regulatory control through conformation changes in proteins, can be found in the fifth chapter. Session VII, devoted to the hormone mediator activity of cyclic AMP, includes also a series of papers.

Though the papers presented compared to the recent papers of pertaining literature, contain rather little that is quite new, this volume makes an attempt to summarize and evaluate results coming from several important fields of metabolic regulation.

ANNA FARAGÓ (Budapest)

RICHTER, G.: **Stoffwechselphysiologie der Pflanzen**
Georg Thieme, Stuttgart (1969), pp. 437, 102 illustrations, DM 12.80.

This work, written in the German language on metabolic physiology, discusses the vital processes of plants. The format of the book is such as to enable the reader to carry it on his person. The style is easy enough to make it intelligible also for persons with but a moderate knowledge of German. The more important parts of the text are accompanied by numerous instructive diagrams and illustrations. The work discusses plant metabolism in six comprehensive chapters. Photosynthesis, its internal and external factors, its structural foundations as well as the role played by environmental physico-chemical factors in the process of photosynthesis are the subjects treated most fully. The large chapter on autotrophy presents a clear picture of photochemical processes (photophosphorylation, quantum requirements, etc.), describes the stages in the reduction of CO_2 as also the synthesis of various carbohydrates. It is in connection with respiration that the catabolic phase of metabolism is discussed most thoroughly. Calling it an important "reservoir" of metabolism, the book presents an instructive description of the citric-acid cycle. Adequately wide space is devoted to the discussion of macromolecules of the protoplasm, such as nucleic acids, proteins, isoprenoids, lipids, etc. Of the esential macroelements, nitrogen, sulphur and phosphorus are dealt with. The concluding chapter treats of water: its molecular structure and dissociation and the water balance of cells. Each chapter contains a brief methodical part. The usefulness of the book is enhanced by a detailed alphabetical index. Not only teachers and investigators engaged in this field but also students of biology, biochemistry, microbiology, pharmacology and other related disciplines may derive profit from studying the well-arranged book.

V. FRENYÓ (Budapest)

THOMAS, J. B.: **Einführung in die Photobiologie**
G. Thieme, Stuttgart (1968), pp. 320, 84 illustr., DM 9.80.

The original work, written in English in 1965, has been translated into German by G. SCHOBER in an exemplary manner.

The author discusses a fundamental subject of biology, one whose progress in the field of molecular biology has been quite as revolutionary as that of genetics.

Three chapters of the first part deal with the fundamental phenomena and basic concepts of photobiology; physical, chemical and biological processes. Beginning with light absorption and the connected state of excitation, the author discusses the electronic energy transfer, the energetics of chemical processes, and the photo-products. After touching in the third chapter on the general question of metabolism, such as the processes of growth, senescence, adaptation and regulation, the author comes to the second, much more extensive, part of the work which treats of biological photophenomena.

The second part contains eight chapters and a concluding chapter. The longest, concerned with photosynthesis, reflects the vast scientific progress achieved in this field in the last decade. The biochemistry of the photosynthesizing pigments and the photoreceptive structures are discussed in detail and on this basis the nature of photosynthetic processes is analysed in detail. It should be noted that the book provides information collected up to 1964.

Chapter 5, dealing with vision, is worthy of especial note. Discussing retinal pigment this chapter, too, starts with biochemical concepts, followed by a comprehensive account of the structures of visual reception; the chapter concludes with an analysis regarding the nature of processes including the theories of colour perception.

A chapter, each, is devoted to the problems of phototaxis, photokinesis, photodinesis, phototropism and the phenomena of photomorphogenesis and bioluminescence.

Some of the biological processes connected with light discussed in the last chapter are usually outside the scope of books of this kind, e.g., the area of U. V. rays, pathological phenomena induced by light, formation of vitamin D, etc. The chapter concludes with an instructive table listing the photobiologically most important pigments and their functions.

The book is provided with an abundant bibliographical list and an exhaustive index.

In summary, the booklet offers an exceedingly instructive picture of the fundamentals of photobiology. It is written in a logical and highly didactic manner. The fact that the work presents the state of knowledge at the level of 1964 does not impair its value since the bulk of essential photobiological information had been collected by that time so that most recent data,

obtained since, refer to details only. The work, a useful guide in the field of photobiology, will surely be read with interest by a wide circle of scientists including plant physiologists, cytologists, biochemists and ophthalmologists.

GY. KISZELY (Szeged)

RAMACHANDRAN, G. N.: **Treatise on Collagen. Vol. 2**
Biology of Collagen in two parts: A and B Ed. by BERNARD S. GOULD. Academic Press, London and New York (1968), Part A: pp. 434, 110s, Part B: pp. 490, 112 s.

This volume deals with different aspects of research on biology, biochemistry and biosynthesis of collagen. From the very first, the selection of contributors is a guarantee for setting down in an excellent manner a large part of our present knowledge as well as the problems still unsolved, a useful tool for those working in this field and particularly for those in related fields. The general biologist will find in these volumes an up-to-date account of the recent developments regarding this fascinating protein.

In Part A: RUSSEL ROSS (Department of Pathology of Washington University) describes "The Connective Tissue Fiber Cell". About "Comparative Biology and Biochemistry of Collagen" the article of K. M. RUDALL (University of Leeds, England), is presented. B. S. GOULD (Division of Biochemistry of Massachusetts Institute), chief editor of this volume, deals with "Collagen Biosynthesis" and with "The Role of Certain Vitamins in Collagen Formation". D. J. JACKSON (Department of Medical Biochemistry, Manchester, England) together with J. P. BENTLEY (Division of Experimental Biology, University of Oregon, U. S. A.) are concerned in "Collagen-Glycosaminoglycan Interaction". The question of "Hydroxyproline and the Metabolism of Collagen" is set forth by D. J. PROCKOP and K. J. KIVIRIKKO (both from the Department of Medicine and Biochemistry, University of Pennsylvania, U. S. A.). The "Mechanical Properties of Collagen Tissues" are described by R. D. HARKNESS (Department of Physiology, University College London, England). D. S. O'DELL (Strangeways Research Laboratory, Cambridge, England) summarizes the present knowledge about "Immunology of Collagen and Related Materials". "The Effect of Hormones on Connective Tissue Cells" is referred by two authors from the Department of Anatomy, University of Utah College of Medicine: T. F. DOUGHERTY and D. L. BERLINER.

In Part B: "The Morphogenesis of Collagen" is presented by SYLVIA FITTON JACKSON (Strangeways Research Laboratory, Cambridge, England). "The Organization and Structure of Bone and the Mechanism of Calcification" is summarized by M. J. GLIMCHER and S. M. KRANE (Massachusetts General Hospital, Boston, Mass., U. S. A.). J. F. WOESSNER JR. (Laboratories for Cardiovascular Research, Miami, Florida, U. S. A.) deals with "Biological Mechanism of Collagen Resorption". A study "On some Aspects of Collagen Formation in Localized Repair and in Diffuse Fibrotic Reactions to Injury" is given by T. GILLMANN (Department of Experimental Pathology, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, England) and "The Role of Collagen in Aging" is disclosed by F. M. SINEX (Boston University School of Medicine, Boston, Massachusetts, U. S. A.).

By all these chapters the authors have attempted to make the treatment extensive for the beginners in collagen field and intensive for the specialists by discussing the latest developments in detail. It is the best up-to-date work on connective tissue research.

ILONA BANGA (Budapest)

Computing in Medicine
British Medical Bulletin **24** (3), Med. Dept. British Council, London (1968). £ 2.

One of the recent issues of the periodical published by the British Council is devoted to the results achieved by the medical and biological use of computers. It is surely unnecessary to dwell upon the significance of the subject since there is nowadays practically no sphere of medical research in which the work of computers could be dispensed with. The manipulation of computers requires adequate training which is, however, often lacking. The issue "Computing in Medicine" facilitates the orientation of beginners and the recognition of existing possibilities by presenting a methodical description of the computer's invasion into the field of medicine. The reader learns the history of computerization, and examples from the sphere of theoretical and clinicopractical investigations acquaint him with its present use in daily practice.

The papers dealing with the fundamental principles of computer technology and the peculiar "language" of computers are particularly instructive. Selection is usefully supplemented by a concise dictionary explaining the meaning of technical terms applied by the booklet in connection with computerization. A similar dictionary of wide range in the Hungarian language would seem to be highly desirable.

In the Introduction, J. A. B. GRAY, professor of physiology, Secretary of the Medical Research Council, emphasizes that the adoption of computers in the field of biological — including the medical — sciences has contributed to the mathematical exactitude of these disciplines: "One of the fields in which computers might well revolutionize biology is in relation to the use of mathematics. Biology in general has always been thought of as the non-mathematical — if not actively antimathematical — part of science. This has arisen because the systems are so complex. Until recently biological measurements were inadequate and mathematical formulations often insoluble."

Computerization has altered facts that were regarded as unalterable. It brought about new facts which have to be understood, accustomed and learnt. Everything that facilitates understanding lightens the researchers' burden and helps in overcoming difficulties. It is by doing so that the "computer" issue of the British Medical Bulletin becomes extremely valuable.

L. VÖRÖS (Budapest)

JACOBS, M. H.: **Diffusion Processes**
Springer Verlag; Berlin—Heidelberg—New York (1967), pp. 160, DM 24.

The book is an English reprint of the successful work published in the series "Ergebnisse der Biologie" in 1935.

The author treats the diffusion processes from two points of view. First a treatment is given based on the theory of heat-conduction in solids, then one based on the molecular structure of matter. He shows that both treatments lead to the same conclusions. The book does no discuss a treatment of diffusion based on thermodynamics.

The author sets out from simplifying presumptions to facilitate the mathematical treatment and for this reason he deals mainly with the different cases of the one-dimensional problem. Another simplifying condition is that the diffusion of a constant amount of substance is considered. First the processes in closed systems are described, then those in systems with one open boundary and finally those in systems with two open boundaries. In the last case one can read about several details (e.g., the case of identical and different boundary conditions or different geometrical conditions). The book works with the concept of diffusion coefficient and gives data to show its dependence upon concentration.

The author deals with diffusion through membranes and emphasizes that this process can be described by relatively simple mathematical methods only in the case of very thin membranes with a homogeneous structure not changing with time. In this connection he points out the difficulties arising when — as in the case of the living organism generally — not only the solute diffuses, but on account of osmotical differences the solvent water too.

The chapter associating diffusion with Brownian movement and containing statements of a general validity exceeding the limits of colloidal systems is also worthy of attention.

In the following the author goes on to the treatment of diffusion processes in more dimensions (in the case of cylinders and spheres) further on to the mathematical interpretation of processes subject to external forces (in gravitational and centrifugal fields) and of diffusion against a convection current. He deals with particular cases of diffusion where the amount of diffusing substance is changing with time. The book ends with a Bibliography of about 16 pages considering the literature up to 1935.

The book assumes only the elements of differential and integral calculus to be known and — correctly — it gives the final relations to be applied without their deduction. It has a great value: drawing the attention to the difficulties rising in the exact mathematical treatment of diffusion processes in the living organism and it shows the possibilities in which cases the calculations can be carried out with small errors.

I. TARJÁN (Budapest

WARE, G. C.: **Basic Electronics for Biologists**
J. A. Churchill Ltd., London (1967) 161 illustrations, pp. 206, 30s.

This book, compiled skilfully and disclosing wide pedagogical experience, obviously would not particularly draw the attention of biologists if it were not bearing the above title, but another referring more closely to its content, e.g., the one proposed by the author in the Preface: "Electronics for the non-electrically minded". The author, a lecturer in bacteriology at the University of Bristol, has based his work upon a series of articles published by him in the journal "Laboratory Practice"; trying to explain to the biologists the working principle of the comparatively simple, most used electronic laboratory apparatuses without any mathematics. This is an aim drawing the attention of not only biologists. Everybody who has to teach physics to non-physicists or non-engineers will fully understand that such a book contains necessarily inaccuracies due to simplification. But as the book contains the text of a lecture on physical subjects held at an English university it is of particular interest for all those, who have attended lectures on biological or similar subjects elsewhere or hold such lectures themselves.

The book is not meant particularly for biologists though it is a work surely interesting for them too. We should need in our university teaching of similar fields books of different type, devoting less attention to basic concepts and some technical details, assuming them to be known. They should deal specifically with the working principle of electronic devices applied in medical research, such as multivibrators, flip-flop circuits, etc.

G. TURCHÁNYI (Budapest)

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CONSIDERATIONS ON THE CHARACTERS OF LIVING ORGANISMS*

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(Received July 12, 1968)

Synopsis

The author attempted to summarize all characters which occur universally in all living organisms. They bear, naturally, definite individual characteristics and thus the universally characterizing features appear as but abstract attributes which, as general ones can be attained only by conceptual means starting from the concrete individual forms. Despite this fact, a recapitulation, serving as a fundamental point of departure for further researches, is deemed necessary.

Introduction

In the classical taxonomy the species is defined on the basis of morphological, embryological, ecological, etc. characters. The number of characters taken into consideration varies from one systematic category to the other conforming to arising necessities. We consider that in all living organisms there are a number of basic characters, necessary to their existence. By the notion "character" we mean in this case the features by which an organism is defined and by which the incorporation of the general into particular is accomplished; the ability of nature to typify, to represent in an individual a historical evolution. The present paper is confined to deal only with characters considered fundamental and necessary for the existence of all living organisms. These characters — due to their universal nature — are abstract ones, from which are derived the concrete characters of the species or of the individual.

Theoretically, the number of characters (N) of an organism could be determined by the formula $N = s \cdot g/r$, where $s =$ the number of the signals carried by a gene, $g =$ the total number of genes, and $r =$ the number of signals necessary for the formation of a character. In view of the facts that our present knowledge does not afford the determination of these values and that certain characters are not carried by a distinct gene — being the result of an interaction between several genes — in listing the characters

*Publishing of pure theoretical papers is generally far off the profile of our journal. As for this paper, however, the Editorial Board makes an exception hoping that its original ideas may deserve a general interest.

we took into account all the characters considered universal, irrespective of their number. In listing them, the bibliography indicated at the end of the present paper was utilized, and attempts were made to formulate each character in a way that it might be applied in the same form to any living organism.

List of abstract characters

The division in groups suggested by us, based partially on the various branches of biological sciences, is merely conventional without corresponding strictly to any objective reality. We maintained it because it might be correlated to earlier classifications of the same kind. In this classification there are several characters which ought to be present simultaneously in a number of groups. We consider that abstract characters may be divided also into the following groups: structural, functional and relational ones; or: spatial and temporal characters; or: substantial, energetic and informational characters; or: characters of individual life and those of perpetuation.

Characters own to the living matter. (1) Material nature, with the primordiality of nucleic acids. (2) Metabolism or character of open system. (3) Growth — expression of the asymmetrical character of the exchanges with the environment. (4) Reproduction — character restoring growth. (5) Biological movement — expression of permanent modifications of living matter and of the actions of those on the medium. (6) Reactivity to environmental changes.

Characters of the organism's aspect. (7) The individual as the fundamental unit in the existence of living matter. (8) The body of the organism as carrier of the individual. (9) Symmetry of the body of the living organism. (10) Existence of a main plane related to the position of the body. (11) The dynamic aspect of the outline of the body. (12) A defined relation between the dimensions of the body. (13) A defined relation between surface and volume of the body. (14) Compactness of the constitution of the body. (15) Segmentation of the body. (16) Aspect of structural localizations. (17) Metameric disposition of the parts. (18) Serial repetition of the parts. (19) Structural polarity of the body. (20) Mono- or polymorphous feature. (21) Sedentary or mobile form. (22) Solitary or colonial form.

Morphological characters of the organism. (23) Morphological type of organization. (24) Mono- or pluricellular (acellular?) differentiation of the organism. (25) Architectonical structure for supporting body weight. (26) Plastic constitutional stability. (27) Elastic constitutional structure. (28) Differentiation of the body cavity. (29) Structure for ensuring the static position of the body. (30) Structure for the fixation of the body. (31) Structure for active or passive locomotion. (32) Structure of selective separation between internal and external medium.

Anatomical characters of the organism. (33) Existence of a nutritional incorporating system. (34) Existence of an eliminatory nutritional system. (35) Existence of a circulatory system. (36) Existence of a system for the exchange of gases. (37) Existence of a functional system of movement. (38) Existence of an information-mediating system. (39) Existence of a relational receptor and analyser system. (40) Existence of a constitutional regenerating system. (41) Existence of a reproductive system.

Physiological characters of the organism. (42) Functions of metabolic coordination. (43) Functions of informational integration. (44) Self-stimulating function. (45) Self-inhibiting functions. (46) Regenerating functions of action energies.

Biocybernetical characters of the organism. (47) Regulatory mechanism of the relations individual—medium. (48) Regulation of vitality by precocity or longevity. (49) Regulation of energy supply and transformation for vital processes. (50) Regulation of homeostasis. (51) Regulation of rhythmical and phasic phenomena. (52) Regulation of bioelectrical potential variations. (53) Regulation of states of rest and action. (54) Regulation of the temporal succession of vital processes. (55) Coordination of the individual development of the organism.

Biomechanical characters of the organism (56) Mechanism for adequation of the centre of gravity. (57) Mechanism for reduction of frictions induced by movement.

Biophysical characters of the organism. (58) State of colloidal aggregation of the living matter. (59) Structural stability of chemical components. (60) Tensions among component chemical particles. (61) Existence of an internal fluid medium. (62) Electrical conductibility of the internal medium. (63) Amplification of connecting information. (64) Processes of molecular disaggregation. (65) Processes of molecular synthesis. (66) Controlled molecular transport. (67) Transmission of bioenergy in living matter.

Bioenergetic characters of the organism. (68) Utilization of external energy sources. (69) Storage of energy. (70) Yield of energy transformations. (71) Speed of energetical processes. (72) Mechanism for decreasing loss of energy.

Biochemical characters of the organism. (73) Elementary constitution of the living matter. (74) Participation in the circulation of elements. (75) Stability of composition of the living matter. (76) Constitutional autocatalyzing duplication. (77) Capacity of synthesis of own substances. (78) Capacity of self-renovation and regeneration. (79) Proteic differentiation. (80) Immunological features of self-protection.

Cytological characters of the organism. (81) Type of cellular functional constitution. (82) Compact or diffuse nuclear constitution. (83) Chromosome number and structure type. (84) Division of hereditary material in reproduction.

Reproductive characters of the organism. (85) Reproductive differentiation. (86) Exchange of reproductive products between individuals or organs. (87) Avoidance of inconvenient reproductive contact. (88) Production of new individuals from the parental body. (89) Temporal succession of generations. (90) Ensuring of the species perpetuation.

Embryological characters of the organism. (91) Formation of the initial cell of the descendant. (92) Nutritional supply of the descendant. (93) Regional inductive specificity of the body of the descendant. (94) Organogenesis of the descendant. (95) Autonomization of the descendant. (96) Ensuring the evolutional stages of the descendant. (97) Attaining of adult stage by growth, development or metamorphosis.

Phylogenetical characters of the organism. (98) Collateral relationship of the organism. (99) Phylogenetical discontinuity of the organism in the history of the species. (100) Space and time coordinates of the organism in the history of the species. (101) The individual, as the actual representative of the history of the species.

Ontogenetical characters of the organism. (102) Limited life span of the organism. (103) Irreversibility of individual development. (104) Succession of developmental periods. (105) Allometrical differentiation in development. (106) Influence of phylogenesis in individual development. (107) Necessity of adequate environmental conditions for development. (108) Effect of influences undergone in the course of development.

Genetical characters of the organism. (109) Existence of hereditary determinism. (110) Capacity of translating genetical code in the structures. (111) Fidelity in the copy of hereditary message. (112) Existence of dominant and recessive characters. (113) Capacity of the segregation of characters.

Genotypical characters of the organism. (114) Correlation of carried characters. (115) Progressivity of carried characters. (116) Transmissibility of carried characters.

Geographical characters of the organism. (117) Influence exerted on the organism by the centre of emergence of the species. (118) Influence of initial dispersion of the species on the organism. (119) Influence of the area of extension of the species on the organism. (120) Influence of geographical isolation on the organism. (121) Influence of the density of individuals on the organism. (122) Influence of migration on the organism. (123) Influence of acclimatization on the organism.

Ecological characters of the organism. (124) Parallelism with the ecosystem in development. (125) Adaptation of ecological valence. (126) Adaptations at trophical level. (127) Rhythmical seasonal adaptations to the abiotic medium. (128) Rhythmical nictemeral adaptations to the abiotic medium. (129) Cyclic adaptations to the biotic medium. (130) Adaptations of competition. (131) Adaptations of survival. (132) Adaptations of adequate be-

haviour. (133) Adaptations for identification among individuals of the same species.

Phenotypical characters of the organism. (134) Capacity of individual manifestation of the characters. (135) Capacity to turn the characters to the account of the individual. (136) Capacity of forming temporal connexions.

Ethological characters of the organism. (137) Preferences for warning. (138) Preferences for collaboration. (139) Preferences for coexploitation of the medium. (140) Preferences for isolation in the biocoenosis. (141) Preferences for aggregation in biocoenosis. (142) Preferences for the occupation of new territories. (143) Preferences for ensuring permanent food supply. (144) Tendency to suprapopulation.

Discussion

This table of characters, considered by the author as universal ones, is far from being complete, due to the fact that it is the first attempt made to list all the common characters of all living organisms as far as it is possible with the information presently available. We tried to give a solid basis to our concept that all living systems, irrespective of their level of organization, display a great number of characters required by their existence. In this connection we shall refer especially to ARISTOTLE who said: "...the form is a common and permanent character for several individuals, accidence is a secondary character which distinguishes individuals ..." DESCARTES' opinion is worth noting too: "I agree with the view advanced by some philosophers claiming that differences of degree exist only in secondary features, but not in the form or intrinsic content of individuals belonging to the same species." In other words, the basic standard of abstract common characters is concretized at the level of each species and each individual to real characters, the complexity of the latter depending upon the level of the given organism.

Analyzing the characters of the above list, we may observe that on the basis of their nature they can be divided into 3 distinct groups: (1) group of abstract existential characters, determining by their presence alone the existence of the organism (for instance characters 1, 2, 7, 33, 61, 68, 109, 126, etc). (2) Group of abstract scalar characters which, besides the fact of existing, might occur in different degrees with changing values (e.g., characters 12, 13, 26, 70, 74, 89, 102, 123, 137, 138, etc). (3) Group of abstract ramifying characters (dycho- or polytomic) where the organisms have the possibility of a choice (e.g., characters 20, 21, 22, 31, 97, etc). Assuming the theory that basic standard of abstract characters in some form common in all organisms we cannot preclude the possibility of the evolution of living systems due to the possibilities existing in the variety of characters of the first group (existential ones)

in a qualitative sense; of those of the second group (scalar ones) in a qualitative and quantitative sense; and of those of the third group (ramifying ones) in a diversifying sense. This variation induced the possibility of a multiple differentiation of concrete characters derived from these abstract ones, ensuring thus the adequation of the organisms to all existing biocoenosis by their adaptive specificity and evolutive complexification.

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I am indebted to Dr. Carol Wittenberger (Biological Research Centre, Branch of Cluj of the Academy of Rumanian R. S.) and to Mr. Ion Paina (Section of Natural History of the "Crişana" museum of Oradea) for their helpful criticism and suggestions, and to my wife for her kind, encouraging support of my study.

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BETRACHTUNGEN ÜBER DIE MERKMALE LEBENDER ORGANISMEN

Es wurde der Versuch unternommen, die Merkmale zusammenzufassen, die allen lebenden Organismen eigen sind. Diese besitzen, natürlich, definitive individuelle Kennzeichen, und deshalb erscheinen die allgemeinen Charakterzüge als abstrakte Attribute, die als allgemeine Kennzeichen nur durch konzeptive Mittel, beginnend von den konkreten Formen erfaßt werden können. Ungeachtet dieser Tatsache wird eine Rekapitulation, die für die weitere Forschung als Ausgangspunkt dienen kann, für wichtig erachtet.

ХАРАКТЕРИСТИКИ ЖИВЫХ ОРГАНИЗМОВ

Автор делает попытку подытожить характеристики, свойственные всем живым организмам. Эти характеристики имеют, естественно, определенные индивидуальные черты и, следовательно, общие характеристики являются лишь отвлеченными атрибутами, которые, будучи всеобщими, достижимы лишь при помощи метода концепции, начиная с конкретными индивидуальными формами. Вопреки этому, авторы считают, что рекапитуляция, служащая исходной точкой для дальнейших исследований, может быть полезной.

CAROL G. NAGY, Marine Research Station, Agigea-Constanţa, Rumania.

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CORRELATION BETWEEN THE CHOLINESTERASE ACTIVITY OF CAPILLARIES AND THE BLOOD—BRAIN BARRIER IN THE RAT

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Synopsis

Inhibition of cholinesterase activity increases the permeability of the blood—brain barrier. The selective inhibition of the activity of either butyrylcholinesterase or acetylcholinesterase has been found to promote macromolecular transportation in the brain of the rat. In the cerebral capillaries of the rat both butyrylcholinesterase and acetylcholinesterase activity can be demonstrated by histochemical methods. The capillaries display acetylcholinesterase activity only over those areas of the brain in which the blood—brain barrier is operative. The two kinds of activity are differently distributed. Butyrylcholinesterase is localized in the pinocytotic vesicles of the cytoplasms of endothelial cells, while acetylcholinesterase activity seems to be restricted to the basement membranes of capillary walls. The butyrylcholinesterase is involved in the regulation of the blood—brain barrier presumably due to its role in transportation, while acetylcholinesterase may act by changing the permeability of the basement membrane.

Introduction

Grieg and Holland [18] have shown that fuchsin, injected into the blood stream, passes into the cerebral tissues under the effect of physostigmine and acetylcholine, indicating that these parasympathomimetic compounds increase the permeability of the blood—brain barrier. Cholinesterase activity in the wall of the cerebral capillaries was described by Koelle [32] in the rat, by Palmer and Ellerker [37] in the sheep. On the other hand, there is — according to our observations and to those of other authors [4, 15, 22, 41] — no butyrylcholinesterase activity in the capillaries over those areas where the blood—brain barrier is inoperative (pineal body, choroid plexus, area postrema, etc.). This applies, according to Dimova and co-workers [7], also to certain other circumscribed cerebral structures (stalk of the pituitary gland, subfornical and subcommissural organs) which, too, are outside the blood—brain barrier. Histochemical methods failed to reveal butyrylcholinesterase activity in the capillaries of these areas.

The present experiments were designed to study the passage of trypan blue across the wall of cerebral capillaries after treatment with specific enzyme inhibitors as used for differentiation of cholinesterase. The employment of such inhibitors in histochemical experiments provides information about the nature of cholinesterase activity in the capillaries of the central nervous system

(CNS). Electron histochemical investigations were performed in order to study the ultramicroscopic localization of butyryl- and acetylcholinesterase.

Material and method

Inbred white rats weighing 180 to 250 g were used. Trypan blue served for visualization of the blood—brain barrier [15]. Observations made by light microscopy showed the method to be unreliable for the study of a possible escape of the dye from the blood circulation. HAMBERGER and HAMBERGER [20] described a sensitive method which, by utilizing the fluorescence of trypan blue, reveals very reliably any change in the permeability of vessel walls following experimental interventions. These authors treated their material by the freezing-drying technique. Since we found that trypan blue retained its fluorescence in cryostat sections, the lesstime-consuming cryostat method was applied in the present experiments. In the control cases we injected 4 to 5 ml of a 1 per cent solution of trypan blue into the tail vein of animals narcotized by the intraperitoneal administration of urethane (1.0 g/kg). Physostigmine (0.25 mg/kg), prostigmine (Stigmosan, 0.25 mg/kg), Mipafox (1.3 mg/kg) and BW 284 C 51 (0.4 mg/kg) served as cholinesterase inhibitors. The drugs were administered intraperitoneally. Thirty minutes after the animals injected with the drugs developed characteristic fasciculation, we narcotized them with urethane (1.0 g/kg) and injected thereafter 4 to 5 ml of 1 per cent trypan blue into the tail vein. Animals were decapited 10, 20 and 30 min after the administration of the dye. Using a cryostat (Mirköz, Budapest), we prepared 20 μ coronal and parasagittal sections of the cerebrum and cerebellum. The preparations were mounted with Entellan and studied under a Zeiss fluorescence microscope; we employed a 200 HBO mercury-vapour lamp with filters BG-12 and OG-1.

The cryostat sections, intended for histochemical examinations, were fixed in 4 per cent formalin for 3 to 5 hours at 4 °C, and the thiocholine technique of KOELLE and FRIEDENWALD [33] as modified by GEREBTZOFF [14] was employed for the demonstration of cholinesterase activity. It is possible to procure a specific inhibition of cholinesterase activity also under histochemical conditions by a pretreatment of the preparations with specific inhibitors [5, 21, 23]. We kept in these experiments the sections for 30 min in 10^{-6} *M* Mipafox or in 10^{-4} *M* Wofatox (methylparathion) or in 10^{-5} *M* BW 284 C 51 at 20 °C. These concentrations of the specific inhibitors were then maintained in the incubating fluid in the further course of the enzyme-histochemical reactions.

For electron histochemistry, perfusion fixation was carried out in a fluid freshly prepared from paraformaldehyde and adjusted to pH 7.4 with 0.14 *M* sodium cacodylate [26]. Other brains were fixed in a buffered solution of formalin–glutaraldehyde [40]. Slices of 3 to 5 mm thickness were fixed in this solution for 3 hours at 4 °C and washed in the buffer overnight. Then 50 μ sections were made by means of a freezing microtome and in order to demonstrate cholinesterase activity by the electron histochemical technique, the sections were incubated according to our "leadthiocholine" procedure [3, 24, 28]. Incubation was followed by treatment with a 2 per cent solution of ammonium polysulphide neutralized by concentrated acetic acid; postfixation was carried out in 1.33 per cent osmium tetroxide buffered with s-collidine [1] for 30 minutes. After dehydration in increasing concentrations of alcohol, the preparations were embedded in Durcupan. Thin sections (silver interference colour) prepared by means of an ultramicrotome of the LKB or the Porter-Blum type, were then studied in an electron microscope, type Tesla 242 D.

Results

Light microscopic histochemical studies

Permeability of the blood—brain barrier can be well studied on cryostat sections by utilizing the fluorescence of trypan blue. Examination after the intravenous administration of the dye revealed that trypan blue — showing a characteristic red fluorescence — had diffused from the capillaries of the choroid plexus (Fig. 1) into the adjacent tissues. The posterior lobe of the hypophysis (Fig. 2) showed the same phenomenon: dye had diffused into the

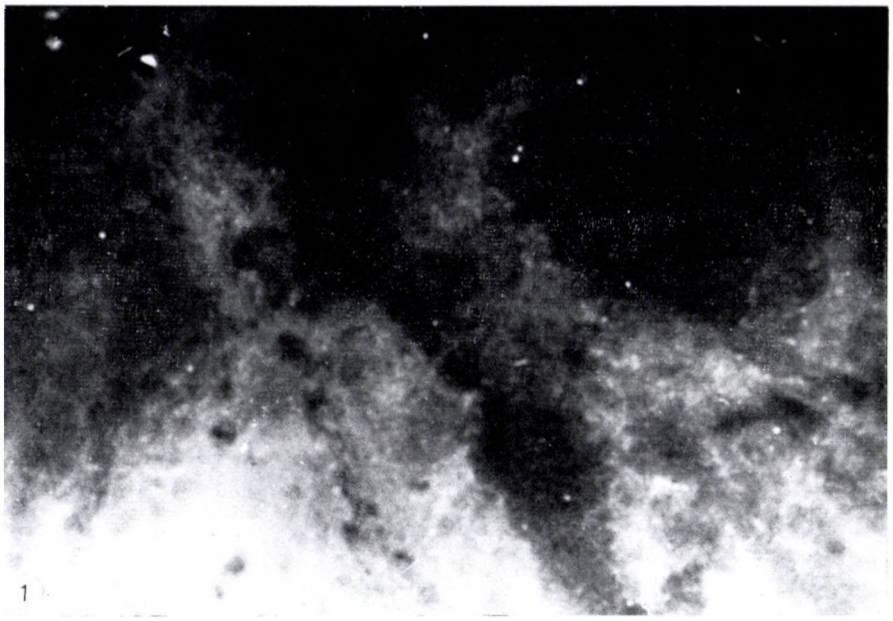


Fig. 1. Choroid plexus 30 min after the intravenous administration of 5 ml 1 per cent trypan blue. Note the penetration of the fluorescent dye into the tissue. × 500

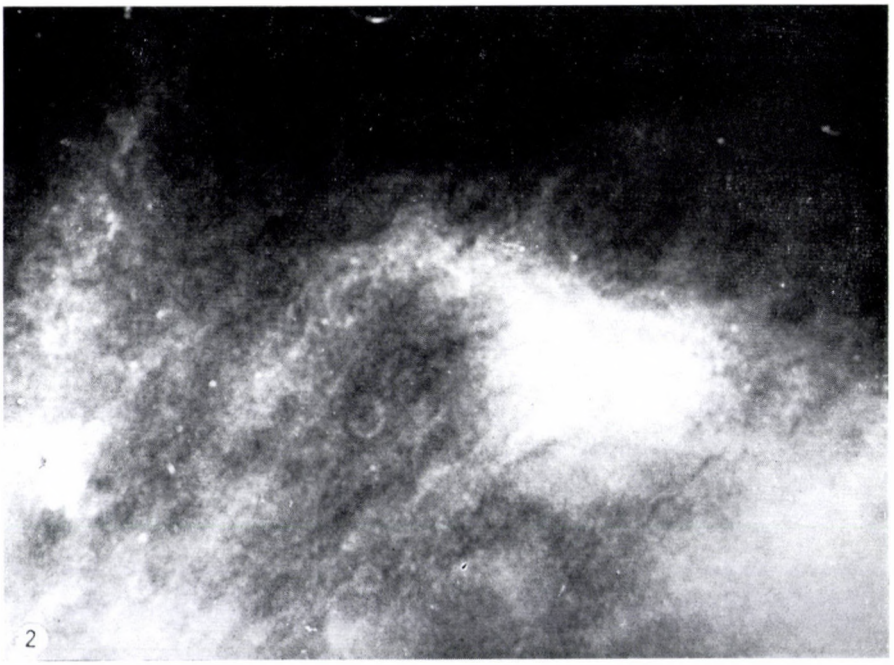


Fig. 2. Detail of neurohypophysis obtained from the animal of Fig. 1. Note extravasation of fluorescent trypan blue and the blurred contour of its diffusion into the tissues. × 500

substance of the neurohypophysis. On the other hand, no such extravasation occurred in the areas protected by the blood—brain barrier: for instance, the dye was found to be evenly distributed in, and produced a sharp relief of, the capillaries of the cerebellar cortex (Fig. 3).

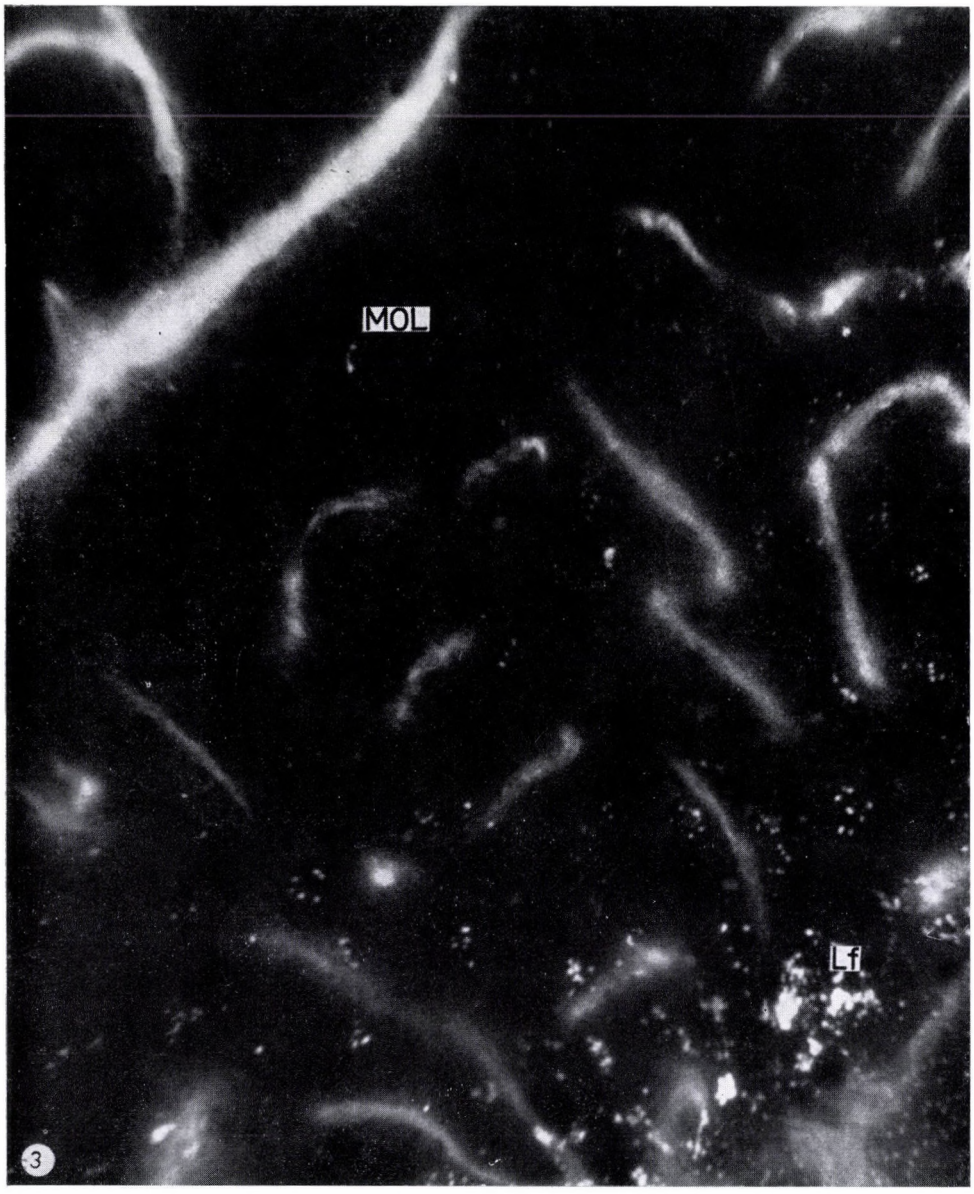


Fig. 3. Cerebellar cortex 30 min after the intravenous injection of trypan blue. No penetration of the dye can be seen in the capillaries of the molecular layer (MOL). Lf = autofluorescent lipofuscin in the cytoplasm of Purkinje cells. × 500

In accordance with literary data [18] we have found with another method that the inhibition of cholinesterase activity by eserine increased the permeability of the blood–brain barrier: dye was able to pass through the vessel walls (Fig. 4). The increase in capillary permeability seems to be in relation to the extent of the perivascular red fluorescence. It has been demonstrated [22, 32] that capillaries in the CNS of the rat show butyrylcholinesterase activity. In agreement with this, pretreatment with Mipafox, a specific inhibitor of butyrylcholinesterase, and in some cases with methylparathion, intensified the passage of trypan blue through the capillary walls in the present experiments (Fig. 5). It was found in earlier histochemical experiments [29] that the cerebral capillaries showed enzymatic activity in some cases also when acetylthiocholine iodide served as substrate. We, therefore, made attempts to influence the permeability of the blood—brain barrier with selective, specific inhibition of the individual cholinesterases. It was found that the intraperitoneally administered BW 284 C 51 (a specific inhibitor of acetylcholinesterase) also suffices for increasing the permeability of the barrier. In the pretreated animals the trypan blue diffused into the cerebral tissues (Fig. 6).

Observations concerning the penetration of trypan blue suggested that increased permeability of the cerebral capillaries following administration of eserine was the result of a summation: capillary permeability was increased, no matter which of two cholinesterases was inhibited. The logical conclusion was that the capillaries of the brain contained both cholinesterases.

In the following histochemical experiments we inhibited butyrylcholinesterase specifically with Mipafox or methylparathion to show whether or not under these conditions the capillaries of the brain exert any cholinesterase activity with acetylthiocholine iodide as substrate. The reaction was positive in both the cerebral cortex (Fig. 7) and the cerebellum (Fig. 8).

The next series of experiments aimed to show whether there existed a topographical relation in the rat brain between the acetylcholinesterase activity of the capillaries and the areas protected by the blood—brain barrier. Therefore, we collected material from those structures which lie outside the blood—brain barrier [4, 15, 41], i.e. the pineal body, the choroid plexus (Fig. 9), the area postrema (Fig. 10) and the subfornical organ. Employment of acetylthiocholine iodide substrate failed to visualize the capillaries of these structures even without specific inhibition with Mipafox or methylparathion, suggesting that as regards the brain capillaries of the rat that also the acetylcholinesterase activity is in close topographical connection with the areas protected by the blood—brain barrier.

Electron microscopic histochemical studies

In harmony with the results of earlier investigations [22, 25] incubation with butyrylthiocholine revealed cholinesterase activity in the cytoplasm

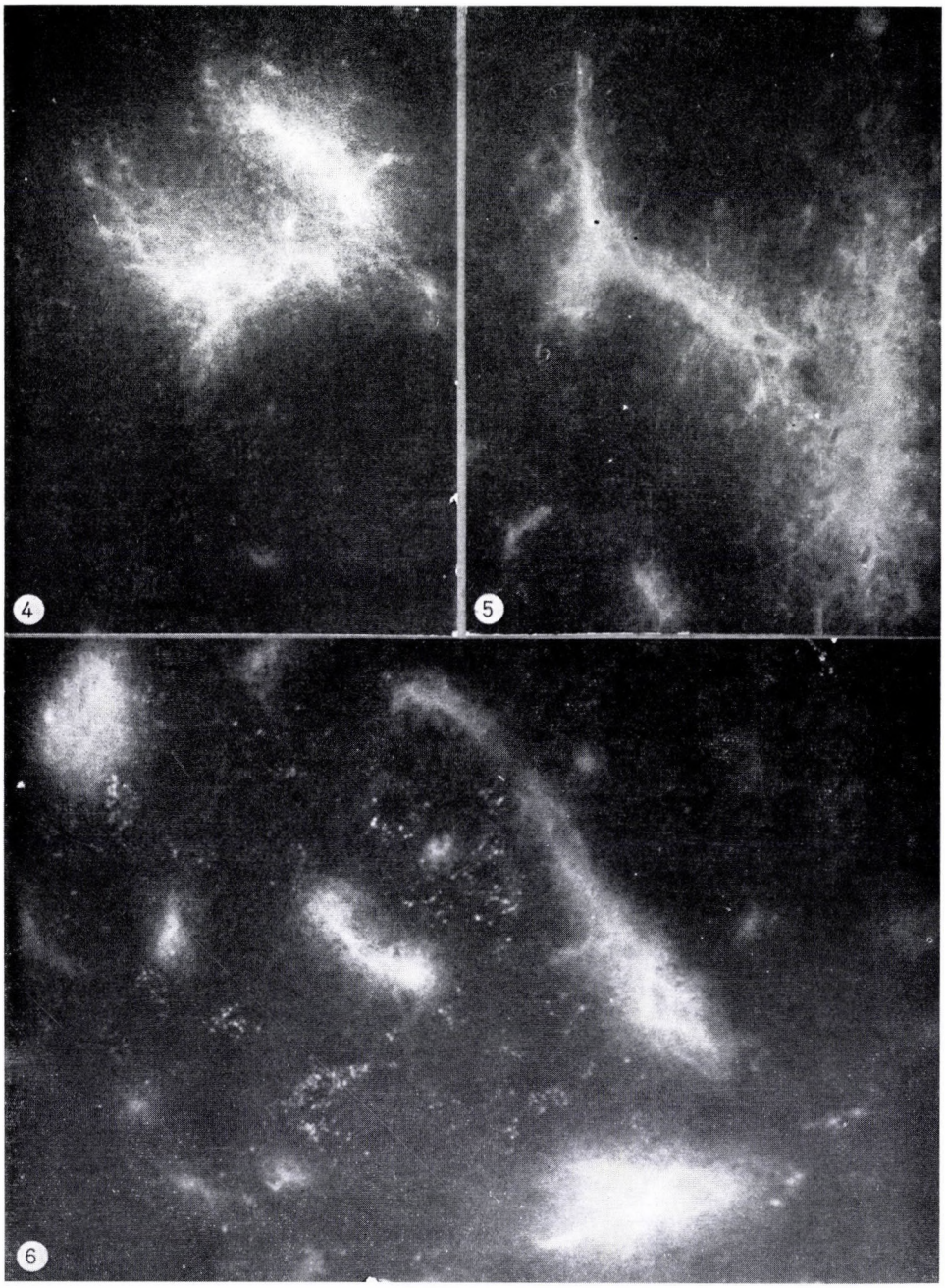


Fig. 4. Penetration around the cerebellar capillaries of trypan blue injected intravenously 30 min after the intraperitoneal administration of physostigmine (0.25 mg per kg). × 500
Fig. 5. Passage through the cerebral capillary of trypan blue injected 30 min after the intraperitoneal administration of Mipafox (1.3 mg per kg). ×500
Fig. 6. Penetration around the cerebellar capillaries of trypan blue injected intravenously 30 min after the intraperitoneal administration of BW 284 C 51 (0.4 mg per kg). ×500

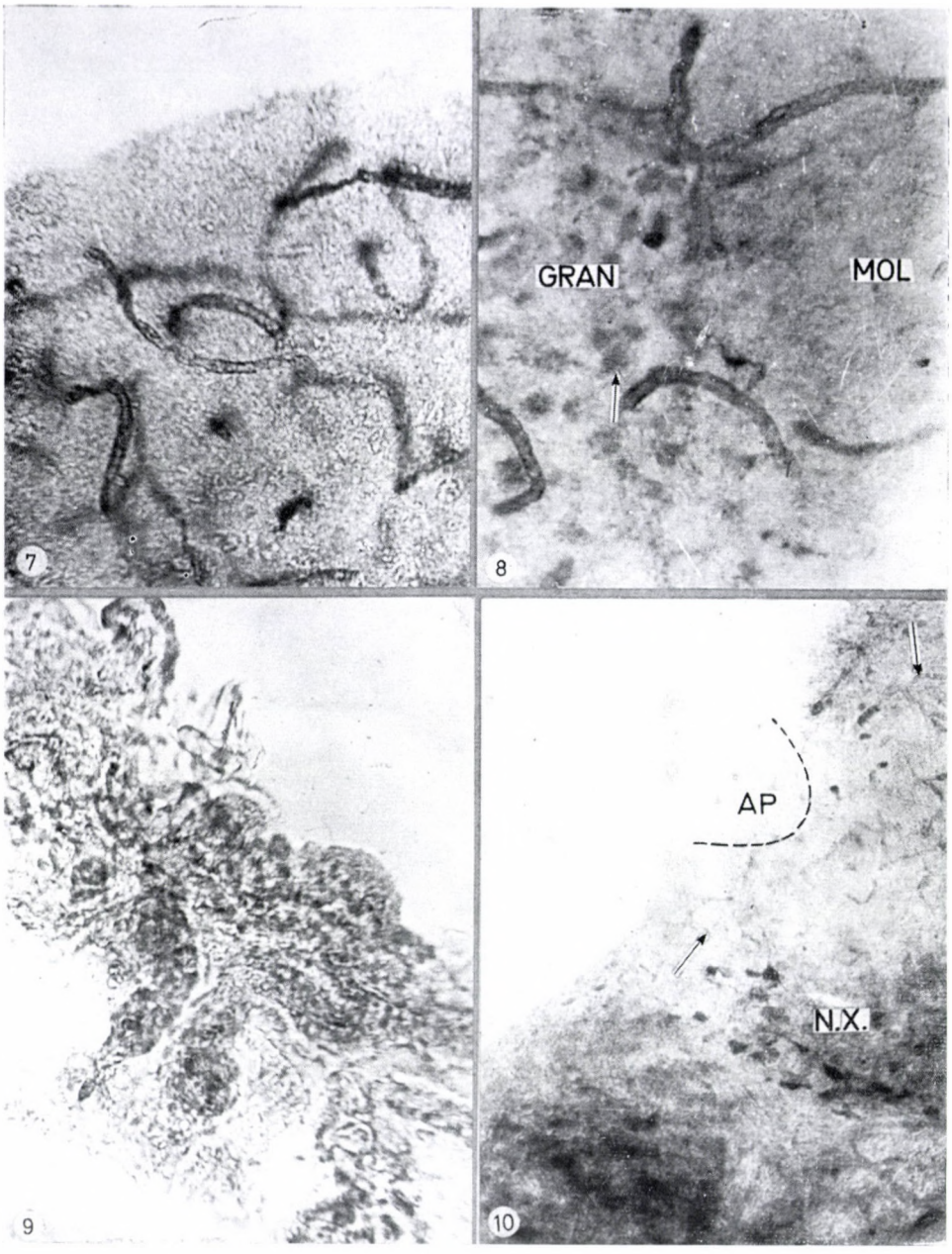
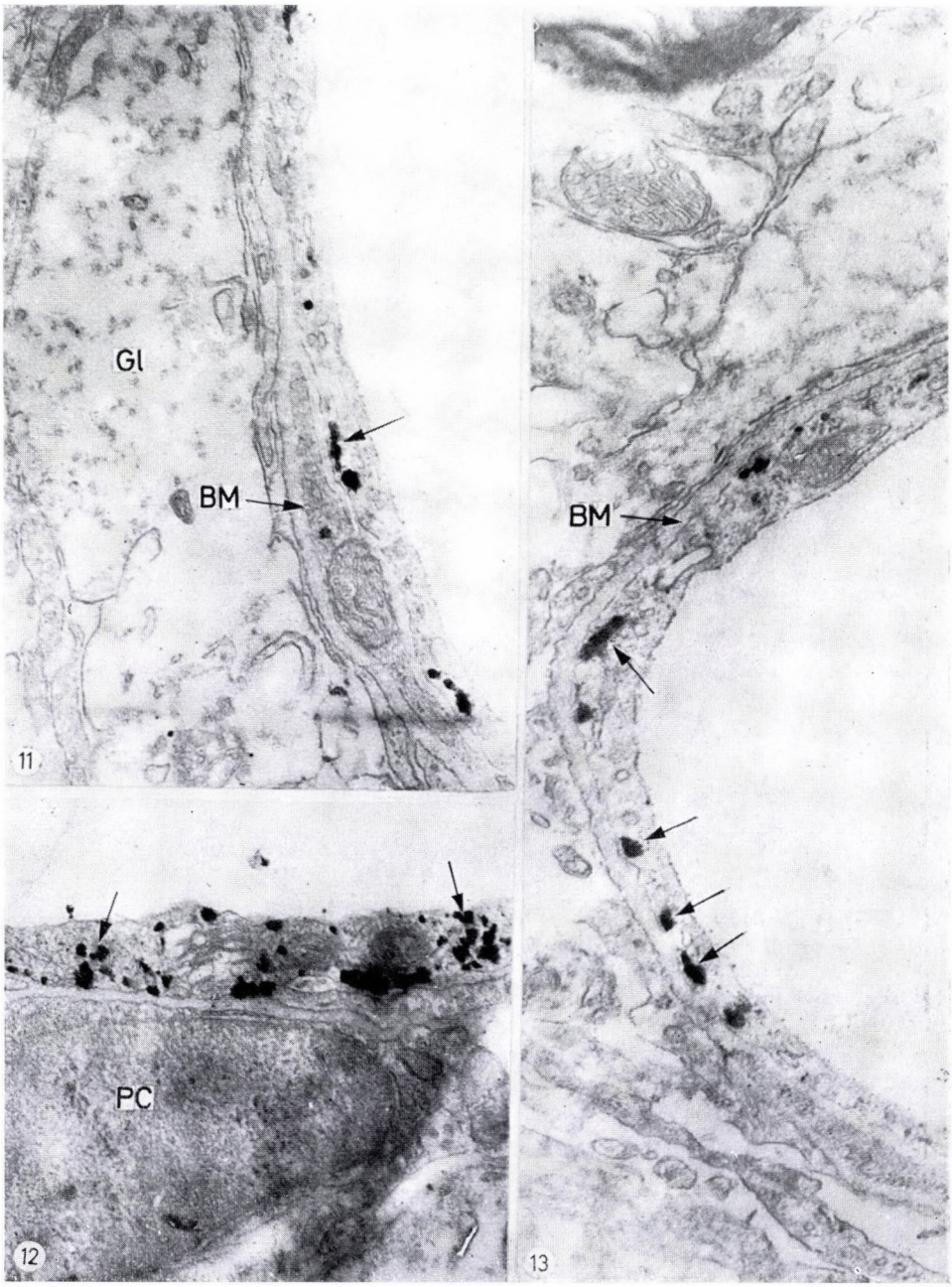


Fig. 7. Cerebral cortex. Capillaries with acetylcholinesterase activity. ×450

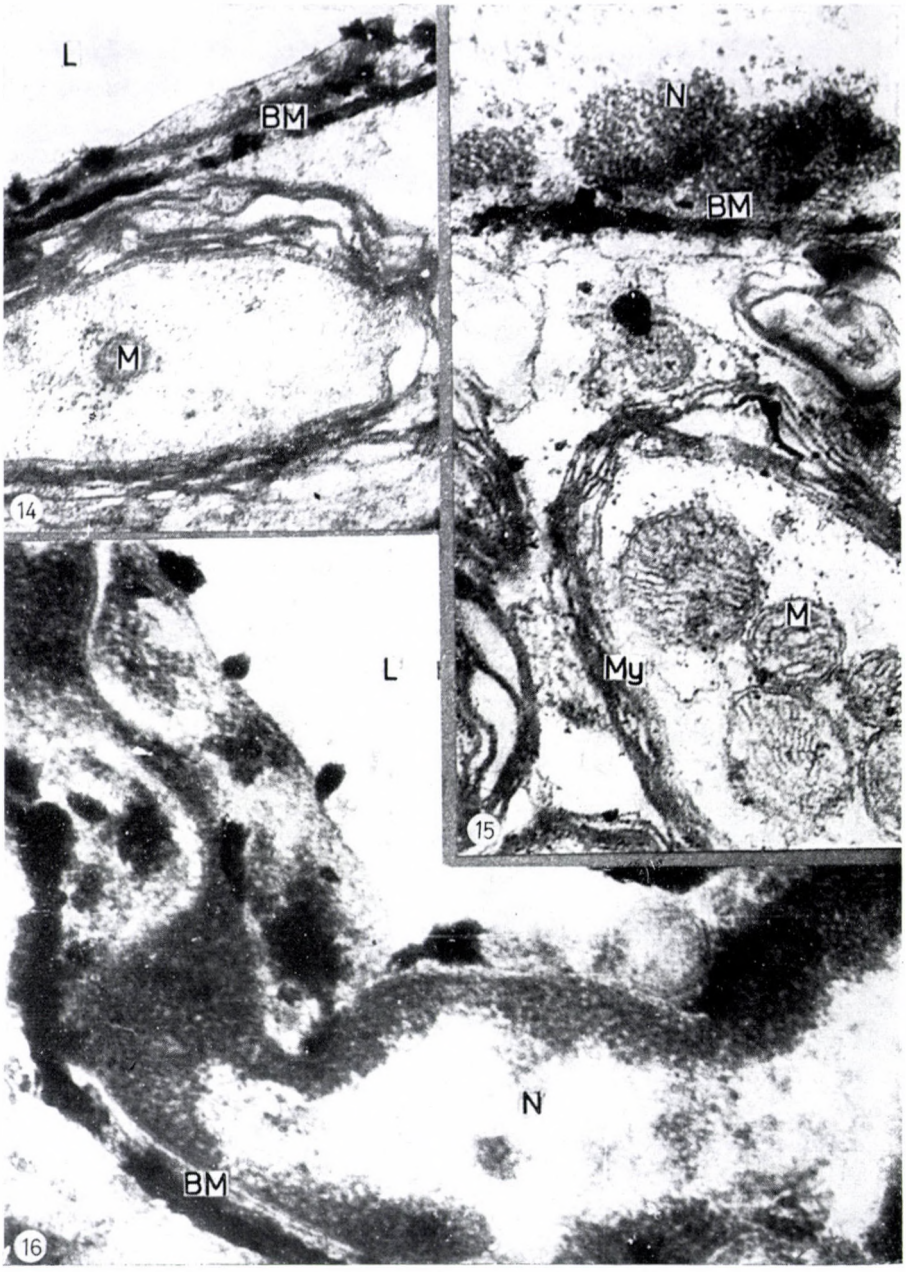
Fig. 8. Cerebellar cortex. Capillaries with acetylcholinesterase activity. GRAN = granular layer, MOL = molecular layer. Arrow points to cerebellar glomeruli containing acetylcholinesterase. ×450

Fig. 9. Choroid plexus. Cholinesterase reaction. No enzymatic activity either in the capillaries or the ground substance. ×450

Fig. 10. Detail of medulla oblongata after acetylthiocholine incubation. Intensive enzymatic activity in the cells of the dorsal nucleus of the vagus (N. X.). The capillaries display cholinesterase activity (arrows). There are no acetylcholinesterase-positive capillaries in the area postrema (AP). ×120



Figs 11—13. Electron histochemical localization of butyrylcholinesterase activity in the capillaries of rat cerebral cortex. The end product of the reaction accumulates in the cytoplasm of endothelial cells, especially in the pinocytotic vesicles (arrows). BM = basement membrane, Gl = glial process, PC = nucleus of a pericyte. ×30,000



Figs 14—16. Electron histochemical localization of acetylcholinesterase activity in the capillaries of the cerebral cortex. The end product of the reaction accumulates in the basement membrane. L = lumen of capillary, BM = basement membrane, M = mitochondria, N = nucleus of endothelial cell, My = myeline sheath. Magnification of Figs 14 and 15: ×30,000; of Fig. 16: ×50,000

of endothelial cells. Electron dense reaction end product was not encountered in the other constituents of the capillaries, in the glial processes adjacent to the basement membrane (Fig. 11) and in the area of the pericytes. It is — provided the time of incubation is optimal — only in the pinocytotic vesicles that enzymatic activity occurred (Figs 12 and 13). Reaction end product attached to structural elements was not obtained in the cytoplasm of the endothelial cells after the procedure employed for the demonstration of acetylcholinesterase activity. Electron dense precipitate was seen in these preparations in the basement membrane of the capillary wall (Figs 14 and 15). Using acetylthiocholine as substrate, we sometimes (especially after long incubation) observed acetylcholinesterase activity in the superficial membrane of the cytoplasm of endothelial cells (Fig. 16).

Discussion

Since the fundamental findings of EHRLICH in 1885 [10] numerous authors have studied the peculiar blood–brain barrier. The barrier function has been attributed to different constituents of the capillaries and of the brain tissue [2, 34]. Relying on the results of electron microscopic examinations, different workers attribute the barrier function (1) to the tight junction of endothelial cells [8, 35]; (2) to the particular structure of the endothelial layer of the brain capillaries [12, 13, 39]; (3) to the basement membrane of the capillaries [36]; or (4) to the astrocytic processes, closely applied to the basement membrane [6, 9].

However, descriptive reports on electron microscopic investigations do not provide reliable information on the question as to which of the constituents of the capillary wall actually acts as a barrier. According to the electron microscopic examinations of GOSZTONYI [16] the endothelial layer alone is enough to prevent the passage of vital stain. Study of the barrier from a morphologico-functional point of view seems a promising way towards a better understanding of the complex operation of the blood–brain barrier. It is known from FLOREY's experiments [11] that intravenously introduced ferritin accumulates in the cytoplasm of the endothelium lining the capillaries of the CNS, further in the pinocytotic vesicles as also in the multivesicular bodies, but does not pass through the basement membrane. On the other hand, REESE and KARNOVSKY [38] demonstrated that the intravenously injected exogenous peroxidase remained in the capillary lumina, indicating that the cytoplasm of endothelial cells functioned as barrier. It might therefore be supposed that substances of different molecular weight are arrested by different constituents of the capillary wall. The result of the two experiments is in any case surprising inasmuch as ferritin with a molecular weight as large as 650,000 [17] is able to reach the basement membrane, while peroxidase with a smaller molec-

ular weight of 40,000 [30] is incapable of even penetrating the cytoplasm of the endothelial cells. Further investigations of this nature might provide additional information about these still unelucidated conditions of permeability.

GRIEG and HOLLAND [18] found the permeability of the blood—brain barrier to have increased following the inhibition of cholinesterase activity by eserine. KELENTEI and FÖLDES [31] supplied quantitative data to show the extent to which parasympathicomimetic drugs influence the permeability of the blood—brain barrier.

By employing specific enzyme inhibitors in histochemical experiments we succeeded in visualizing the cerebral capillaries with acetylthiocholine iodide as substrate after the selective inhibition of butyrylcholinesterase. It follows that *both butyrylcholinesterase and acetylcholinesterase are active in the capillaries of the rat's CNS, further, that — as regards activity of both butyrylcholinesterase and acetylcholinesterase — the cerebral capillaries are in a close topographical connection with the areas protected by the blood—brain barrier.*

According to histochemical studies performed with specific enzyme inhibitors by GWIN and WOLSTENCROFT [19], the capillaries in the floor of the fourth ventricle contain both butyrylcholinesterase and acetylcholinesterase in the cat, whereas only cholinesterase is contained in the other areas of the medulla, e.g. in the capillaries of the inferior oliva. We, too, observed [27] that, with acetylthiocholine iodide substrate, cholinesterase activity was demonstrable in the capillaries of the cerebellar cortex of the cat; no inhibition was, however, obtained by means of BW 284 C 51, whereas Mipafox produced marked inhibition. It seems, therefore, that both butyrylcholinesterase and acetylcholinesterase are invariably co-existing in the capillaries of those areas of the rat's CNS which are protected by the blood—brain barrier, whereas some capillaries of the cat brain contain these cholinesterases together and others exert acetylcholinesterase only.

With regardrs to the localization of cholinesterase activity in the ultramicroscopic structure of the cerebral capillaries, it was demonstrated in earlier experiments [22, 25] that butyrylcholinesterase activity in the brain of the rat was present in the cytoplasm of endothelial cells, especially in the pinocytotic vesicles, while acetylcholinesterase activity occurred in the basement membrane of the capillary vessels. KÁSA and CSILLIK [28] found acetylcholinesterase in the basement membrane of the capillary wall in the cerebellum of the cat.

All the above experiments prove that the capillaries of the rat's CNS contain both butyrylcholinesterase and acetylcholinesterase. It has been found that permeability of the blood—brain barrier is increased irrespective of whether the activity of the one or that of the other cholinesterase is specifically inhibited. While both forms of cholinesterase are present in the capillaries protected

by the blood—brain barrier, no cholinesterase is contained in the cerebral capillaries of unprotected structures. The electron microscopic examinations have shown that the two cholinesterases are differently distributed in the structures of the capillary wall. The presence of butyrylcholinesterase in the cytoplasm and the pinocytotic vesicles suggests that — in connection with its participation in the transportation of particulate matter — this enzyme is somehow involved in the function of the blood—brain barrier. Again, the presence of acetylcholinesterase in the basement membrane of the capillary walls makes it probable that this enzyme acts on the blood—brain barrier by regulating the permeability of the basement membrane.

Investigations are now in progress to ascertain the possible effect of other enzymes of the cerebral capillaries on the permeability of the blood—brain barrier.

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ZUSAMMENHANG ZWISCHEN DER CHOLINESTERASE-AKTIVITÄT DER KAPILLAREN UND DER HÄMATOENCEPHALISCHEN BARRIERE IM RATTENHIRN

Nach einer Lähmung der Cholinesterase-Aktivität mit Enzyminhibitoren läßt sich eine erhöhte Permeabilität der hämatoencephalischen Barriere beobachten. Nach selektiver Hemmung sowohl der Butyrylcholinesterase- als auch der Acetylcholinesterase-Aktivität findet im Rattenhirn ein Anstieg des makromolekularen Transports statt. Mit histochemischen Verfahren läßt sich in den Kapillaren des Rattenhirns neben der Butyrylcholinesterase-Aktivität auch eine Acetylcholinesterase-Aktivität aufzeigen. Die Kapillaren besitzen eine Acetylcholinesterase-Aktivität nur in jenen Hirngebieten, in denen auch eine hämatoencephalische Barriere vorhanden ist. In der feinstrukturellen Lokalisation der Butyrylcholinesterase- und der Acetylcholinesterase-Aktivität ist ein Unterschied wahrnehmbar. Die Butyrylcholinesterase-Aktivität ist in den pinocytotischen Bläschen des Cytoplasmas der Endothelzellen, die Acetylcholinesterase-Aktivität hingegen in der Basalmembran der Kapillarwand lokalisiert. Es wird angenommen, daß die Butyrylcholinesterase über ihre Teilnahme am Stofftransport, die Acetylcholinesterasen-Aktivität durch die Veränderung der Basalmembran-Permeabilität an der Steuerung der Funktion der hämatoencephalischen Barriere beteiligt ist.

СВЯЗЬ МЕЖДУ ХОЛИНЭСТЕРАЗНОЙ АКТИВНОСТЬЮ КАПИЛЛЯРОВ И ГЕМАТОЭНЦЭФАЛИЧЕСКИМ БАРЬЕРОМ В ГОЛОВНОМ МОЗГЕ КРЫСЫ

После торможения холинэстеразной активности ингибиторами энзимов повышается проницаемость гематоэнцефалического барьера. После избирательного торможения как активности бутирилхолинэстеразы так и активности ацетилхолинэстеразы в головном мозге крысы наблюдается увеличение макромолекулярного транспорта. При исследовании гистохимическими методами в капиллярах головного мозга крысы кроме бутирилхолинэстеразной активности выявляема также ацетилхолинэстеразная активность. Капилляры показывают также ацетилхолинэстеразную активность лишь в тех областях головного мозга, в которых существует гематоэнцефалический барьер. В микроструктурной локализации бутирилхолинэстеразной и ацетилхолинэстеразной активностей наблюдается разница. Активность бутилирхолинэстеразы локализуется в пиноцитотических везикулах цитоплазмы эндотелиальных клеток, в то время как активность ацетилхолинэстеразы локализуется в базальной мембране стенки капилляров. Полагается, что бутирилхолинэстераза участвует в регулировании функции гематоэнцефалического барьера путем участия в транспорте веществ, а активность ацетилхолинэстеразы — путем изменения проницаемости базальной мембраны.

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EFFECT OF LARVAL CUTICLE ON THE ACTIVATION OF PHENOLOXIDASE IN DROSOPHILA MELANOGASTER

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Synopsis

The cuticle of the late third instar-larvae was found to contain a phenoloxidase activating substance. This cuticular substance strongly promoted the activation of the proenzyme contained in the haemolymph. The haemolymph of larvae contains an activator of non-cuticular origin. The activator of cuticular origin was found to activate also proenzyme derived from unpigmented pupae. The cuticular activator is sensitive to heat, it is presumably a protein. The larval cuticle shows no phenoloxidase activity. The reduction of phenoloxidase activity observed at the time of pupation is probably correlated with the sclerotization of the larval cuticle, a phenomenon which counteracts the effect of the cuticular activator.

Introduction

Numerous data have been published on the phenoloxidase activity of insects. The problems of melanin formation [24] and sclerotization [13] have been investigated most thoroughly.

The o-diphenoloxidase (E. C. 1.10.3.1. o-diphenol: oxygen oxidoreductase, also called dopaoxidase, phenolase or phenoloxidase) is present as inactive proenzyme in the haemolymph of numerous insects *(Calliphora, Musca)*. The proenzyme is activated by protein or lipoprotein activators. Its activation can be induced by various chemicals, such as surface-active compounds and heavy metals [7].

Horowitz and Fling [6] have shown that fresh extracts of *Drosophila* have no phenoloxidase activity and that a certain time of incubation is necessary to elicit enzyme activity. This finding applies to the successive stages of ontogenesis also [20].

Several alternative hypotheses have been advanced regarding the complex mechanism of activation, suggesting that it results from an autocatalytic process [6] or from a limited proteolysis of the proenzyme [12], or an aggregation of subunits [21] leads to the appearance of the active enzyme.

None of these hypotheses has yet been convincingly proved by experiments. The common feature of the hypotheses is the postulation of several components supposed to be involved in the development of the active enzyme.

Horowitz and Fling [6] suggest the participation of at least two protein components, while others [21] suppose the presence of five or even more [22] components.

Attempts have been made with a view to locating the proenzymes and activators and to reveal their nature. The presence of diphenoloxidase was demonstrated by DENNEL [3] in the larval cuticle of *Sarcophaga falculata* and by LAI-FOOK [15] in *S. bullata*. In *Calliphora erythrocephala* prophenoloxidase was found in the larval haemolymph and the activator in the larval cuticle [11]; the latter was proved to be an enzyme.

Drosophila melanogaster has been examined for phenoloxidase activity [16, 23]. It appears that, while the haemolymph contains the proenzyme (or proenzymes), the activator(s) as well as the other components necessary for the formation of the active enzyme are located in other tissues and organs.

The elucidation of problems regarding diphenoloxidase in *Drosophila* is difficult because at least two mechanisms may be responsible for the production and activity of the enzyme, namely genetic control and regulatory factors [17].

The present study had the object to ascertain the presence of, and examine the role played by, the activator supposed to be contained in the cuticle of *Drosophila melanogaster* larvae.

Material and method

Larvae and pupae of *Drosophila melanogaster* CyL mutant were used. The larvae were kept in a thermostat at 25 $\pm$ 0.5 °C on Belgovsky's nutrient medium.

Preparation of enzyme. Late third instar-larvae as also pupae were repeatedly washed in Ringer solution in order to remove the nutrient medium. The animals were then homogenized in a 20-fold volume of *M*/15 phosphate buffer of pH 6.8 at 0 °C. The homogenate was thereafter centrifuged at 0 °C with 20,000 g for 10 minutes. The enzyme was contained in the supernatant fluid.

When preparing the haemolymph we modified the procedure: the larvae were squashed in the buffer and passed through gauze; the filtrate was then centrifuged, the supernatant serving as haemolymph preparation.

Preparation of the cuticular activating factor. Having washed third instar-larvae in Ringer solution, we squashed them between sheets of filter paper in order to remove the haemolymph and the internal organs. The remaining cuticle was repeatedly washed, dried between filter papers, homogenized in a tenfold volume of phosphate buffer and centrifuged as described above. The activator was contained in the supernatant fluid.

The diphenoloxidase enzyme was activated by incubation at 0 °C. Enzyme activity was measured by determining the formed dopachrome photometrically at 480 mμ, 25 °C, in a Spectromom 202 photometer. A 0.02 *M* solution of 3,4 DL-dihydroxyphenylalanine (dopa) served as substrate. The mixture of the reaction contained 3.0 ml of dopa and 0.4 to 0.8 ml of extract (corresponding to 1 mg), diluted to 1.0 ml with phosphate buffer. Enzyme activity was expressed in optical density per mg protein units as a function of time. Protein was determined by LOWRY's method [19].

Results

Proenzyme and activator contents of the larval cuticle are illustrated in Fig. 1. The cuticular extract of the larvae displayed no phenoloxidase activity even after long incubation indicating that the cuticle is devoid of this enzyme.

The haemolymph extract required 120 min of incubation to reveal marked activity. The activation increased gradually until the 360th minute and remained constant thereafter. The activation of the haemolymph extract was considerably influenced by the extract of the cuticle. The rate of activation showed a sudden rise and attained a much higher value than haemolymph extract alone.

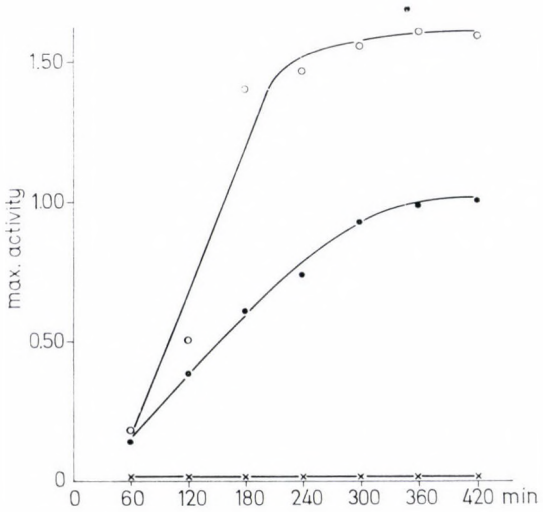

Fig. 1. Maximum phenoloxidase activity as a function of the incubation time: larval haemolymph (●); haemolymph containing cuticular extract (○); cuticular extract ×)

Results justify the conclusion that the cuticle of third instar-larvae of *Drosophila* contains the activator (or activators) of phenoloxidase; this substance potentiates the activation of the proenzyme *in vitro*. This is, however, not the only activator, since the haemolymph extract which contained no cuticular components was likewise activated by "endogenous" non-cuticular activator. However, the course of the activation process was in this case quite different from that described in the foregoing (Fig. 1).

Phenoloxidase activity is known [22] to reach a peak before pupation and to decrease thereafter very considerably. Renewed increase of enzyme activity occurs only about 60 hours after pupation. We wanted to find out the cause of the low level of enzyme activity at the time of pupation: whether the proenzyme or the activator is lacking at that stage.

Collecting pupae between the 20th and the 30th hour following pupation, we measured their phenoloxidase activity and examined the effect of larval cuticle extracts. Results are shown in Fig. 2.

It was found that, with reference to the larval incubation time, the time of incubation necessary for the development of full activity (lag period)

was cut down to 120 minutes at the pupal stage and that the level of maximum activity was lower.

Treatment with the extract of larval cuticle increased the rate of activation, and, compared to the pupal extract, its highest values showed an approximately 100 per cent increase, a phenomenon indicative of the fact that the activator of the larval cuticle was operative in this case also. The maximum activity was still considerably inferior to the value obtained in the larvae, suggesting that the proenzyme is also deficient during pupation.

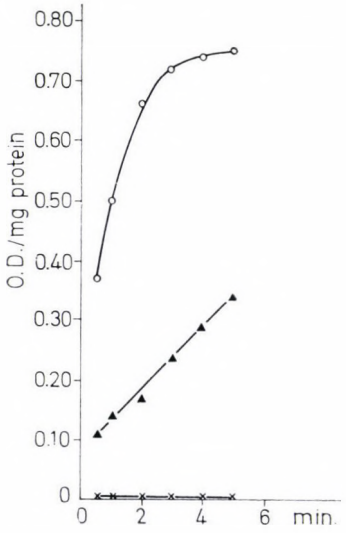

Fig. 2. Effect of extract of larval cuticle on the phenoloxidase activity of an extract prepared from unpigmented pupae. Incubation: two hours at 0 °C: cuticle (×); extract of unpigmented pupae (▲); enzyme activity of incubated 1 : 1 mixture composed of larval cuticle extract [4.2 mg protein/ml] and pupal extract [2.22 mg protein/ml] (○)

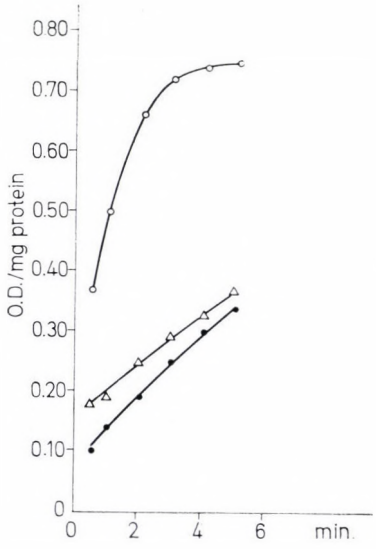

Fig. 3. Behaviour of activator effect of larval cuticle extract after exposure to heat: activity of heat-treated cuticular extract and pupal extracts [1 : 1 mixture] (△); activity of non-heated cuticular extract and pupal extracts [1 : 1 mixture] (○); activity of control 1 : 1 mixture of buffer and pupal extract (●); incubation: two hours at 0 °C

Investigations into the nature of the cuticular activator yielded contradictory results. Certain authors do [2], others do not [5] regard it as being of protein nature. In order to elucidate this question we incubated the cuticular extract at 100 °C for five minutes and examined thereafter its activating effect on extracts prepared from unpigmented pupae. Results are shown in Fig. 3.

It can be seen that incubation at 100 °C resulted in a practically total loss of activating power; the observed activity was hardly different from that of buffer and pupal extracts used as controls. The difference between the two extracts may be explained by the observation made by ASHIDA and OHNISHI [2] on *Bombyx mori*. The larval cuticle contains, according to these authors, also other than protein activators. If the activating factor is precipitated by alcohol from the cuticular extract of the *Drosophila* larvae, and re-dissolved, it will still retain its activating power. The activating substance is not dialysable. It is presumably of protein nature.

Discussion

The number and function of proteins contained in the phenoloxidase "complex" of insects are still rather obscure. The proteins in question may be proenzymes, activators or other substances with unknown functions. The phenoloxidase activity is demonstrable in the *Drosophila melanogaster* only from the third larv alstage. This is thought to be due to that all proteins except one needed for the production of the active enzyme are synthesized at an earlier larval stage; the biosynthesis of one protein takes place at the third stage only [19].

It has been demonstrated [4] that a special protein (protein S) is produced by the salivary gland of *Drosophila* which component may have a catalytic role in the activation of phenoloxidase. This is supported by the present study: protein S may have gained access from the salivary gland to the extract in the course of the preparation of haemolymph and thus promoted activation. That also other than cuticular substances are responsible for activation seems to be beyond doubt.

Certain authors [18, 22] suggest the possible presence of a lipoidal activator in *Drosophila*, while others [2] found that the activators contained in the cuticle of *Bombyx mori* larvae consisted mostly of protein, although non-protein activator(s) too must be present, because dialysis diminished the activator effect. That the substance contained in the cuticle of *Lucilia cuprina* retained its activating power during a 5-minute incubation at 80 °C speaks against its protein nature. It seems evident that the cuticular factor plays an important part in activation, but a non-cuticular or non-proteinic factor may also be involved in the process.

According to KARLSON and co-workers [10, 14, 25] the proenzyme occurs in the haemolymph, moves into the cuticle in the course of pupation and becomes an active enzyme; quinones are produced which react with the enzyme which takes part in sclerotization. This would mean that the activator in the larval cuticle and part of the proenzyme in the haemolymph of *Drosophila* may turn into inactive proteins in the course of sclerotization.

According to our observations, chiefly the lack of activator is responsible for the low phenoloxidase activity.

Experiments with the cuticular extract of *Drosophila* larvae have yielded results different from those obtained from experiments with *Sarcophaga bullata* [15] or *Musca domestica* [5] in which phenolases were demonstrated in the cuticle. These deviations may be due to differences in the test objects.

The problem of phenoloxidase function is further complicated by the fact that there exist presumably various forms of this enzyme which may turn into each other [8, 9]. The different forms possess different activities towards mono- and diphenols.

In vitro examinations concerning the mode of action of phenoloxidase have so far not sufficiently revealed the processes which occur in living insects, e.g. it has been shown that no correlation exists between the *in vitro* phenoloxidase activity of extracts and the pigmentation of *Drosophila* mutants [17].

The role played by phenoloxidase in the pigmentation and sclerotization of insects requires further investigations.

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DIE WIRKUNG DER LARVALEN KUTIKULA AUF DIE PHENOLOXYDASE-AKTIVIERUNG BEI *DROSOPHILA MELANOGASTER*

Verfasser untersuchten den Aktivator des in der larvalen Kutikula von *Drosophila melanogaster* vermuteten Phenoloxydase-Enzymsystems und erwogen auch die Frage, ob die larvale Kutikula, neben dem Aktivator, Phenoloxydase in Form eines Proenzyms oder aktiven Enzyms enthält. Die Kutikula der im späten 3. Larvenstadium befindlichen Larven enthält eine Substanz von Aktivatornatur. Der kutikuläre Aktivator beeinflußt in hohem Maße die Aktivierung des in der Hämolymphe enthaltenen Proenzyms. Die Hämolymphe der Larven enthält auch einen Aktivator nichtkutikulärer Herkunft. Der aus der larvalen Kutikula stammende Aktivator übt auch auf das aus der unpigmentierten Puppe extrahierbare Proenzym eine aktivierende Wirkung aus. Der kutikuläre Aktivator besitzt aller Wahrscheinlichkeit nach Eiweißcharakter und verliert unter Wärmeeinfluß seine Aktivatoreigenschaft. Die larvale Kutikula besitzt keine Phenoloxydase-Aktivität. Auf Grund dieser Befunde ist es wahrscheinlich, daß die bei der Einpuppung beobachtete Verminderung der Phenoloxydase-Aktivität mit der Sklerotisation der larvalen Kutikula in Zusammenhang steht, die zur Inaktivierung des kutikulären Aktivators führt.

ДЕЙСТВИЕ ЛИЧИНОЧНОЙ КУТИКУЛЫ НА АКТИВАЦИЮ ФЕНОЛОКСИДАЗЫ У *DROSOPHILA MELANOGASTER*

Авторы изучали активатор энзиматической системы фенолоксидазы, предположительно присутствующий в кутикуле личинки *Drosophila melanogaster*, а также вопрос, содержит ли личиночная кутикула, кроме активатора, фенолоксидазу в форме проэнзима или активного энзима. Было установлено, что в кутикуле личинки в поздней 3-й личиноч-

ной стадии имеется вещество природы активатора, в значительной мере влияющее на активацию проэнзима, присутствующего в гемолимфе. В гемолимфе личинок имеется также активатора не кутикулярного происхождения. Активатор из кутикулы личинки оказывает активирующее действие и на проэнзим, получаемый из гомогенной куколки. Кутикулярный активатор имеет, по всей вероятности, белковую природу, и под влиянием тепла он теряет свое активаторное свойство. Кутикула личинки не обладает активностью фенолоксидазы. На основе полученных данных кажется вероятным, что наблюдаемое при закукливании уменьшение активности фенолоксидазы связано со склеротизацией кутикулы личинки, приводящей к инактивации кутикулярного активатора.

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MORPHOLOGICAL BACKGROUND OF THE REGULATION OF NEPHRIDIAL ACTIVITY IN THE HORSE LEECH (HAEMOPIS SANGUISUGA L.)

STUDY OF NEPHRIDIAL INNERVATION BY MEANS OF ESTERASE REACTION

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Synopsis

The innervation of the nephridia has been studied in the horse leech on the evidence of esterase activity. The nephridia are provided with a rich network of nerves whose fibres terminate on or between the cells of the tubules. Innervation in the urinary bladder is restricted to the mural muscles. As regards the origin of nephridial nerves, a large multipolar nerve cell was observed beside each nephridium near the boundary between the nephridial canal and the urinary bladder, and these nerve cells play an important role in nephridial innervation. It is concluded that nephridial activity is neurally regulated.

Introduction

Nephridial structure and function have long been studied by a great many investigators [3, 4, 5, 10], whereas literature is poor in data dealing with the regulation of nephridial activity. ROOTS [14] demonstrated that in certain earthworms the concentration of the surrounding medium affects the rate of activity of the nephridiostome cilia. According to KAMEMOTO [5], in the earthworm the osmotic value and ionic composition of the coelomic fluid are influenced by the neurosecretion of the brain. It is possible that this phenomenon is due to nephridial activity.

We have found no reliable data in the available literature regarding the neural regulation of nephridial activity. Although significant observations have been made concerning the innervation and neural regulation of the intestinal canal of annelids [1, 2, 12, 13], the impregnation method employed in these studies involves considerable technical difficulties [1, 2].

We have found that the peripheral nerves of the horse leech show esterase activity. For this reason in the present study we have attempted to elucidate the problem of nephridial innervation by histochemical demonstration of esterase activity.

Material and method

Nephridia of the horse leech (*Haemopis sanguisuga* L.) were examined for esterase activity. Freezing sections fixed by formalin were subjected to the following histochemical procedures.

(1) Naphthylacetate-azodye method with L-naphthylacetate substrate and o-dianisidine diazonium salt.
(2) Indigo dye with 5-bromindoxylacetate substrate.
(3) Determination of cholinesterase by acetylthiocholine iodide.
(4) Determination of cholinesterase by thioacetic acid.

To determine the origin of fibres innervating the nephridial tubules, certain segments of the abdominal ganglionic chain were removed and subjected to the above listed histochemical procedures after one to fourteen days.

The common medicinal leech (*Hirudo medicinalis* L.) has proved to be unsuitable for the purposes of this study.

Results

The central nervous system and the peripheral nerves of the horse leech stained intensively with the naphthylacetate-azodye (Fig. 1), whereas in the medicinal leech esterase activity was strong in the ganglia only. Therefore, only the horse leech could be used in the present experiments. The peripheral nerves of the horse leech, even their finest ramifications, stain dark brown or black with naphthylacetate-azodye and can therefore be well studied in the tissues of low esterase activity. Although the central nervous system reacted intensively to treatment with indigo and acetylthiocholine iodide, the peripheral nerves were not easily identifiable because of their less intensive and granular staining. Treatment with thioacetic acid failed to yield evaluable results.

Esterase activity in the nephridial tubules of the horse leech was moderate at certain points, but generally rather weak. Intensive staining reaction made the nerve fibres clearly distinguishable in the tubular area (Figs 2 to 4). The nephridial tubules were found to be covered by a rich network of nerves. The thicker fibres, running to and ramifying on the surface of the nephridium, sent off thin branches (Figs 2, 3). The (sometimes slightly cirsoid) fibres of the terminal branches were found to end on or between the epithelial cells of the nephridial canal. Running between these cells, the thin fibres extended almost to the lumen of the tubules (Fig. 4). There was, as regards innervation, no essential difference between the various parts of the nephridial canal. It seemed, on the other hand, impossible to demonstrate an innervation of epithelial cells in the wall of the urinary bladder; the muscles were, however, frequently accompanied by thin nerve fibres which probably terminate on them (Fig. 5).

Following the removal of a part of the abdominal ganglionic chain the pattern of innervation in the nephridia of the same segment remained unchanged even after two weeks. It is, therefore, improbable that nephridial nerves have a direct central origin. Careful examinations have revealed the existence of just a single nerve cell per nephridium which seemed to be involved in nephridial innervation. It was situated near the boundary between the nephridial tubule and the urinary bladder. The multipolar cell measured 50 to 60 μ, and esterase activity was intensive both in its body and processes (Figs 6, 7). Although the course of the processes is still somewhat obscure,

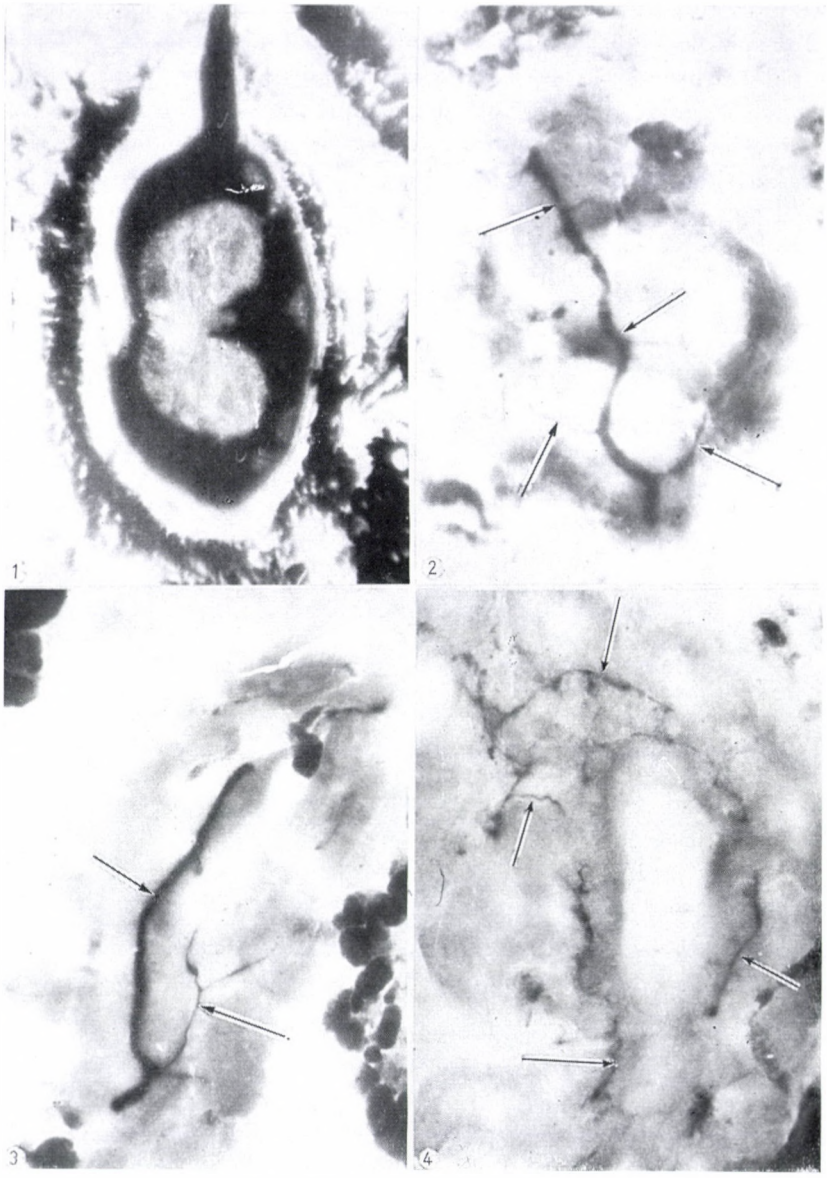

Fig. 1. Abdominal ganglion of *Haemopis sanguisuga*. Naphthylacetate-azodye. Intensive esterase activity in the neurones and neuroglia cells at the periphery of, as well as in the nerve emerging from, the ganglion

Figs 2—4. Innervation of the nephridial canal of *Haemopis sanguisuga*. Arrows point to innervating fibres. Naphthylacetate-azodye

they are undoubtedly involved in nephridial innervation. The cell in question is usually spindle-shaped; a thick process was found to arise from each of its two poles. These processes extend over a fairly long distance, and part of their branches run to more remote portions of the nephridium (Fig. 7), while the termination of other branches has not yet been elucidated. A few thin processes, arising from the side of the cell, run to and ramify in the adjacent nephridial tubules (Figs 6, 7).

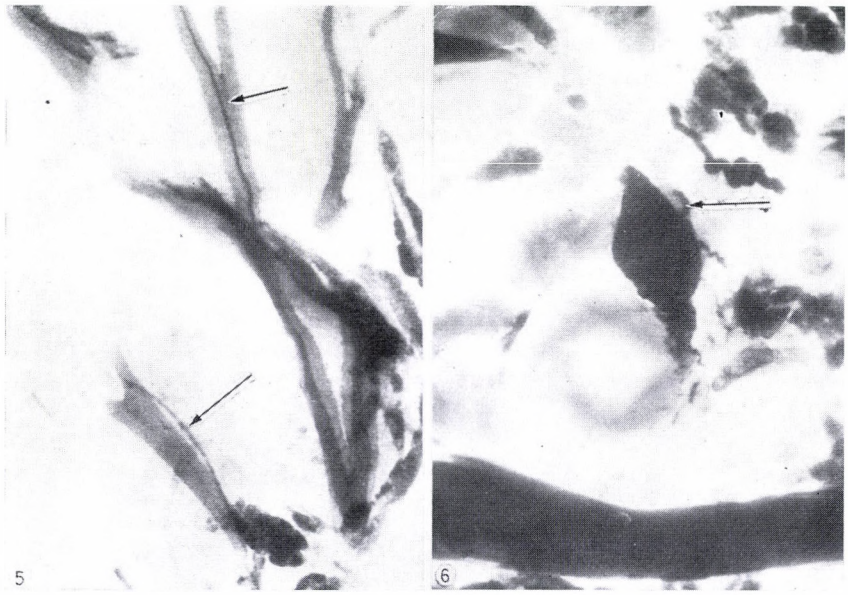

Fig. 5. Arrows point to fibres innervating muscles in the urinary bladder of *Haemopis sanguisuga.* Naphthylacetate-azodye

Fig. 6. Nephridial nerve cell of *Haemopis sanguisuga.* Arrows point to processes running to a nephridial tubule. Naphthylacetate-azodye

Discussion

Innervation of the kidney in vertebrates is fairly well known, whereas the innervation of excretory organs in the Invertebrata is a neglected subject of comparative neurohistology. We failed to encounter reports on the nephridial innervation of the Annelida: the lack of data is presumably due to technical difficulties. The horse leech proved to be a satisfactory test object in this respect: the intensive esterase activity of its nephridial nerves makes it amenable to adequate histochemical examinations. By demonstrating the rich innervation of the nephridial epithelium and the vesical muscles we have paved the way for further investigations concerning the neural mechanism of nephridial

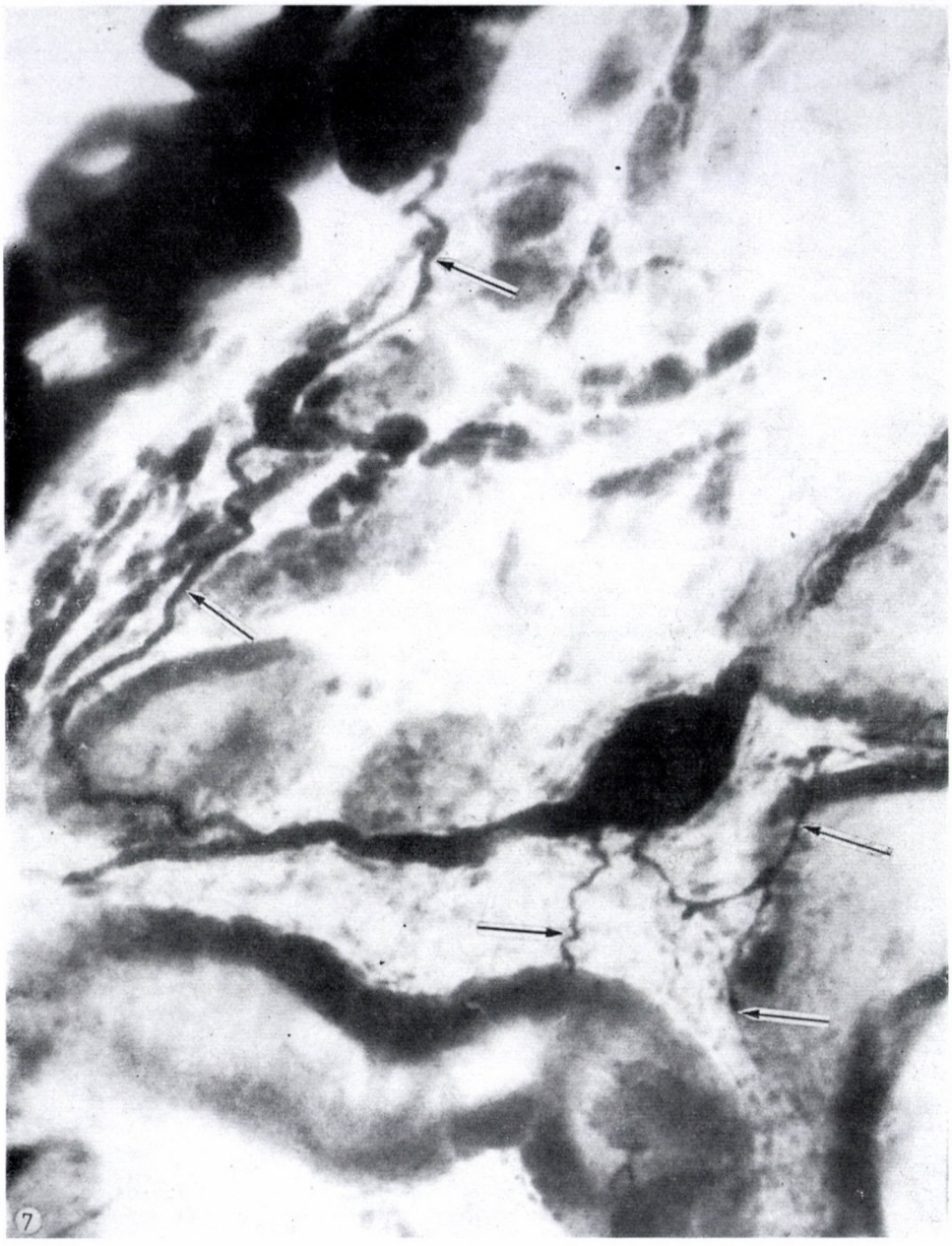

Fig. 7. Nephridial nerve cell of *Haemopis sanguisuga*. Arrows point to processes running to a nephridial tubule. Naphthylacetate-azodye

regulation. We regard the observed single neurone as responsible for a direct innervation of each nephridium. Conditions of innervation are, thus, simple enough for the nephridia of *Haemopis* to serve as models in investigations concerned with the neural regulation of excretion.

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DIE MORPHOLOGISCHEN GRUNDLAGEN DER REGULATION DER NEPHRIDIENFUNKTION IM ORGANISMUS DES PFERDEEGELS

Untersuchung der Nephridieninnervation mit Hilfe der Esterasereaktion

Die Innervation der Nephridien der Egel wurde mit histochemischer Methodik, gemessen an der Esteraseaktivität, untersucht. Die Nephridienkanälchen sind von einem reichen Nervengeflecht durchzogen, deren Fasern an den Zellen des Kanälchens oder zwischen ihnen endigen. In der Harnblasenwand beschränkt sich die Innervation auf die Muskeln der letzteren. In bezug auf den Ursprung der Nephridiennerven wurde festgestellt, daß sich neben jeder Nephridie, nahe der Grenze des Nephridialkanals und der Harnblase eine große multipolare Nervenzelle befindet, der in der Innervation der Nephridien eine wichtige Rolle zukommt. Demnach steht die Nephridialfunktion unter neuraler Regulation.

МОРФОЛОГИЧЕСКИЕ ОСНОВЫ РЕГУЛЯЦИИ ФУНКЦИИ НЕФРИДИЙ В ОРГАНИЗМЕ ПИЯВОК (*HAEMOPIS SANGUISUGA* L.)

Изучение иннервации нефридий измерением эстеразной активности

Гистохимическим методом исследования изучалась иннервания нефридий пиявок на основе эстеразной активности. Нефридиальные канальцы переплетены нервным сплетением, волокна которого оканчиваются на клетках канальца или между последними. В стенке мочевого пузыря иннервация распостраняется лишь на мышцы стенки мочевого пузыря. Относительно происхождения этих нервов было установлено, что возле каждого нефридия, около границы нефридиального канальца и мочевого пузыря, располагается крупная мультиполярная нервная клетка, которая играет важную роль в иннервации нефрий. Следовательно нефридиальная функция находится нейральной регуляцией.

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INHIBITION OF INCREASED PRODUCTION OF LUTEINIZING HORMONE IN CASTRATED AND ANDROGENIZED RATS

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Synopsis

Further evidence of a double central effect of neonatal testosterone treatment ("androgenization") is published. Accordingly, a large dose (500 μg) of androgen, subcutaneously injected on the first day of extrauterine life, (1) desensitizes the sex-hormone-sensitive neurones of the anterior hypothalamus and (2) damages, in the hypophyseotrophic area, the structures producing the GTH-releasing factors. The method of parabiosis and male detector rats was used to determine the degree of LH production. The accessory genital organs of animals joined with castrated controls grew in weight more than twofold during the 12 days of parabiosis, whereas the partners of castrated androgenized rats displayed no change in comparison to non-operated control animals. It is concluded that the production of LH is not increased in neonatally androgenized rats after the elimination of the feed-back mechanism.

Introduction

As regards the effect of neonatal androgen treatment the conclusions of FLERKÓ and co-workers [4, 5, 6, 19, 20, 21] are in contradiction to those of MORRISON and JOHNSON [18]. While according to FLERKÓ and co-workers hypothalamic structures otherwise sensitive to sex steroids are desensitized, MORRISON and JOHNSON suggest that the hypothalamo-hypophyseal system is made more sensitive to the inhibitory action of sex steroids. Of recent, BOYD and JOHNSON [3] have pregnantly stated: "... androgenization of females did not cause differentiation of a 'male type' hypothalamus but rather differentiation of a hypothalamus exquisitely sensitive to androgen." This statement contradicts, among others, our observations [13, 16] indicating that neonatal androgenization results in a 'male type' differentiation of the hypothalamo-hypophyseal system not only as regards GTH production but also respecting the production of prolactin and somatotrophin.

We have shown [15] that the experimental results of MORRISON and JOHNSON [18] allow another interpretation if a double central effect of neonatal androgenization is taken into account. It is suggested by BARRACLOUGH [1, 2] that, while small doses of androgen act merely on the preoptic area, large doses may also act on the arcuate and ventromedial nuclei, changing the threshold value of their activity. Relying on the evidence of our findings [14, 15] we suppose that neonatal androgen treatment exerts a double central effect: (1) in the anterior hypothalamus the structures which are sensitive to sex

steroids are desensitized, (2) in the hypophyseotrophic area the neural structures that are responsible for the primary secretion of GTH are damaged [7, 8]. As a consequence, the production (secretion and release) of FSH and LH is likewise reduced. This is the most pronounced effect of testosterone administered on the first day of extrauterine life.

The present experiments were designed to demonstrate that castration of rats androgenized by a large dose of testosterone administered immediately after birth does not increase LH production.

Material and method

A total of 152 white inbred rats ("OKI-Budapest" strain) of Wistar origin were used. Five-hundred μg testosterone phenylpropionate dissolved in 0.1 ml oil (Retandrol-RICHTER, Budapest) was administered subcutaneously to part of littermate male and female animals on the first day after birth; the rest served as controls.

Littermate animals of identical body weight were joined parabiotically between the 40th and 50th day of life; part of the animals were castrated at the same age.

The pairs were severed in ether anaesthesia along the line of suture on the 12th postoperative day. Then the animals were weighed, decapitated and dissected. The endocrine organs (hypophysis, thyroid gland, adrenal, testicles and accessory genital glands) were weighed, using a torsion balance, with an accuracy of ± 0.2 mg. The accessory genital organs were first weighed together; the seminal vesicle and the prostate were then separated, the semen was expelled and blotted. The two organs were then separately weighed so that the amount of the sperm could be calculated. The following abbreviations and symbols are used in the Tables and the text:

C ♀ = control female
C ♂ = control male
A ♀ = androgenized female
A ♂ = androgenized male
CC = castrated control
CA = castrated androgenized animal.

The animals were divided into the following experimental groups:

Group 1 : single C ♂
Group 2 : C ♂ + A ♂
C ♂ + A ♀
Group 3 : C ♂ + CC ♂
Group 4 : C ♂ + CA ♂
Group 5 : C ♂ + CC ♀
Group 6 : C ♂ + CA ♀
Group 7 : single A ♂
Group 8 : A ♂ + C ♀
A ♂ + A ♂
A ♂ + A ♀
Group 9 : A ♂ + CC ♂
Group 10 : A ♂ + CA ♂

Statistical significance of differences was determined according to Student's "t" test [10].

Results

Results are presented in four Tables. Comparison of the figures regarding "single" animals (Tables 1 and 2) shows that the testicle and accessory genital glands of not "parabiotized" androgenized animals were smaller than those

Table 1

Weight of testicle and accessory glands of control male rats after a 12-day parabiosis with intact and androgenized castrated animals

Group	Partner	Number of cases	Weight of animals (g)	Weight of testicle (mg)	Total weight of accessory glands (mg)	Weight of seminal vesicle (mg)	Weight of prostate (mg)	Sperm*** (mg)
1. Single C ♂	—	9	153.5 ± 2.8*	1900 ± 70	322.0 ± 31.7	96.4 ± 2.8	196.0 ± 3.8	29.6
2. C ♂	A ♂ A ♀	8	127.2 ± 7.2	1618 ± 112	337.0 ± 35.2	105.0 ± 13.3	178.7 ± 20.9	53.3
3. C ♂	CC ♂	10	135.1 ± 7.2	1855 ± 99	739.4 ± 182	199.5 ± 36.7	283.5 ± 57.2	256.4
4. C ♂	CA ♂	14	132.8 ± 3.2	1725 ± 110	330.0 ± 53.9	109.0 ± 15.7	144.0 ± 20.8	77.0
5. C ♂	CC ♀	4	141.6 ± 18.0	2012 ± 222	515.0 ± 114	139.0 ± 27.7	212.0 ± 12.8	164.0
6. C ♂	CA ♀	6	110.5 ± 11.4	1726 ± 169	229.0 ± 52.9	69.9 ± 15.1	100.5 ± 10.2	58.6
					1—2	$P > 0.5$**	$P > 0.5$	
					3—4	$P < 0.02$	$P < 0.02$	
					5—6	$P < 0.05$	$P < 0.02$	

* standard deviation
** probability
*** difference between total weight and the weight of accessory tissues

Table 2

Weight of testicle and accessory glands of androgenized male rats after a 12-day parabiosis with intact and androgenized castrated partners

Group	Partner	Number of cases	Weight of animals (g)	Weight of testicle (mg)	Total weight of accessory glands (mg)	Weight of seminal vesicle (mg)	Weight of prostate (mg)	Sperm*** (mg)
7. Single A ♂	—	17	152.2 ± 1.5*	1162 ± 32.9	150.6 ± 4.9	56.5 ± 2.4	94.1 ± 3.3	0.0
8. A ♂	A ♂♀ A ♀ C ♀	7	127.6 ± 7.2	1142 ± 149	138.2 ± 15.6	44.2 ± 8.2	87.0 ± 10.4	7.0
9. A ♂	CC ♂	7	146.2 ± 6.5	1131 ± 198	375.9 ± 62.6	110.0 ± 16.3	193.0 ± 32.2	72.9
10. A ♂	CA ♂	7	141.0 ± 6.8	1100 ± 245	173.0 ± 31.3	62.0 ± 12.6	88.0 ± 12.0	23.0
					7— 8	P > 0.5**	P > 0.5	
					8— 9	P < 0.01	P < 0.01	
					8—10	P > 0.5	P > 0.5	
					9—10	P < 0.05	P < 0.01	

* standard deviation
** probability
*** difference between total weight and the weight of accessory tissues

of the untreated controls of the same age. The difference was statistically significant ($p < 0.01$).

In the non-castrated group, C ♂ and A ♂ animals were parabiotically united with A ♂, A ♀ or C ♀ partners. After a parabiosis of 12 days neither the endocrine organs of the control males nor those of the androgenized male animals exhibited signs that would have indicated any essential change in GTH production.

In further groups one of the parabiotic partners was castrated. As a result of this, the weight of the accessory genital glands was found to have considerably changed, but the testicles did not differ in weight in any of the combinations from the values found in the corresponding "non-parabiotic" animals or in parabiotic animals having uncastrated partners.

It is clear from Table 1 that the prostate and seminal vesicle of the C ♂ animals parabiotically united with CC ♂ weighed considerably more than those of control males joined together with non-castrated partners. (Seminal vesicle: 105 vs. 199.5 mg; prostate: 178.7 vs. 283.5 mg.) Increase in the weight of the accessory genital glands was considerably less if the parabiotic partner was not only castrated but androgenized as well. (Seminal vesicle: 109 vs. 199.5 mg; prostate: 144 vs. 283.5 mg; $p < 0.02$.) The accessory genital glands of the detector C ♂ considerably increased in weight if it was in parabiosis with CC ♀ animal. (Seminal vesicle: 105 vs. 139 mg; prostate: 178.7 vs. 212 mg.) On the other hand, no hypertrophy was registered if the partner was both castrated and androgenized. A statistically significant weight difference in the accessory genital organs was observed in detector animals having CC ♀ and CA ♀ partners. (Seminal vesicle: 105 vs. 139 and 69.9 mg, respectively; prostate: 178.7 vs. 212 and 100.5 mg, respectively.)

Table 2 shows the results of experiments in which the detector animal was androgenized. The accessory genital organs of A ♂ animals parabiotically united with CC ♂ animals grew twofold in comparison to those having uncastrated partners; the difference was significant: $p < 0.01$. (Seminal vesicle: 44.2 vs. 110; prostate: 87 vs. 193 mg.) There was hardly any change in the weight of the accessory genital organs of the androgenized detector male if it had a CA ♂ animal, i.e. a castrated and androgenized male animal as partner. Weight increase was significantly less in these cases than in the partners of CC ♂ animals.

It is quite evident from Tables 1 and 2 that the hypertrophy of the accessory genital glands of detector male animals was considerable if their partners were CC but hardly demonstrable if they were CA. In none of the groups did the testicles show any essential change.

Table 3 shows the above data in a different arrangement. We wished to compare C ♂ and A ♂ animals, united with identically treated parabiotic partners, as regards testicular hormone production and the reactivity of the

Table 3

Comparative weights of testicle and accessory glands of control and androgenized male rats after a 12-day parabiosis with intact and androgenized castrated partners

Group	Partner	Number of cases	Weight of animals (g)	Weight of testicle (mg)	Total weight of accessory glands (mg)	Weight of seminal vesicle (mg)	Weight of prostate (mg)
2. C ♂	A ♂ A ♀	8	127.2 ± 7.2*	1618 ± 112	337.0 ± 35.2	105.0 ± 13.3	178.7 ± 20.9
3. C ♂	CC ♂	10	135.1 ± 7.2	1855 ± 99	739.4 ± 182	199.5 ± 36.7	283.5 ± 57.2
4. C ♂	CA ♂	14	132.8 ± 3.2	1725 ± 110	330.0 ± 53.9	109 0 ± 15.7	144.0 ± 20.8
8. A ♂	A ♂ A ♀ C ♀	7	127.6 ± 7.2	1142 ± 149	138.2 ± 15.6	44.2 ± 8.2	87.0 ± 10.4
9. A ♂	CC ♂	7	146.2 ± 6.5	1131 ± 198	375.9 ± 62.6	110.0 ± 16.3	193.0 ± 32.2
10. A ♂	CA ♂	7	141.0 ± 6.8	1100 ± 245	173.0 ± 31.3	62.0 ± 12.6	88.0 ± 12.0

* standard deviation

accessory genital organs. The effect of GTH passing from the parabiotic partner CC ♂ was approximately the same on both the C ♂ and the A ♂ detectors: the weight of the examined organs increased about twofold, while the presence

Table 4

Weight of the hypophysis, thyroid gland and adrenals of single and parabiotized (12 days) control and androgenized male rats

Group	Partner	Number of cases	Weight of animals (g)	Weight of hypophysis (mg)	Weight of thyroid (mg)	Weight of adrenals (mg)
1. Single C ♂	—	9	153.5 ± 2.8	6.8 ± 1.0	11.9 ± 2.2	27.8 ± 6.3
7. Single A ♂	—	17	152.2 ± 1.5	6.6 ± 0.1	12.5 ± 0.2	36.6 ± 0.5
2. C ♂	A ♂ A ♀	8	127.2 ± 7.2	5.5 ± 0.3	11.9 ± 1.2	34.4 ± 1.8
3. C ♂	CC ♂	10	135.1 ± 7.2	5.5 ± 0.3	11.2 ± 2.5	34.0 ± 1.4
4. C ♂	CA ♂	14	132.8 ± 3.2	5.1 ± 0.3	10.8 ± 0.7	36.6 ± 1.6
5. C ♂	CC ♀	4	141.6 ± 18.0	5.9 ± 0.4	9.0 ± 0.6	37.6 ± 9.4
6. C ♂	CA ♀	6	110.5 ± 11.4	4.7 ± 0.5	8.9 ± 1.0	43.0 ± 5.2
8. A ♂	A ♂ A ♀ C ♀	7	127.6 ± 7.2	5.8 ± 0.1	10.7 ± 0.3	41.1 ± 5.5
9. A ♂	CC ♂	7	146.2 ± 6.5	6.3 ± 0.3	11.6 ± 0.6	40.5 ± 4.0
10. A ♂	CA ♂	7	141.6 ± 6.8	6.6 ± 0.1	10.8 ± 0.6	45.9 ± 2.6

of a castrated and androgenized (CA ♂) partner did not, or did hardly, change the size or secretory activity of the accessory genital organs.

The weights of endocrine glands of "non-parabiotic" and detector animals are shown in Table 4. The figures bear out the observation that parabiosis does not induce essential changes in this respect. Increase in the weight of the suprarenal glands may be ascribed to surgical stress and immune reaction.

Discussion

Administration of androgen to newborn male rats decreases the weight of both testicles and accessory genital organs [1, 13, 25]. Testosterone if given on the first day of life is more efficacious than if administered on the third. The significantly lower weights point, in themselves, to a reduced hypophyseal production of GTH and/or to a decreased sensitivity of the target organs to hormones. However, the results of the present experiments seem to prove that in androgenized male rats neither the sensitivity of the testicle to GTH nor that of the accessory genital glands to androgens decreases. The weight of the accessory genital organs of androgenized detector male animals (A ♂) increases at same rate as does that of C ♂ animals under the effect of the GTH following from the CC partners (from 138 to 375 mg = 270 per cent and from 337 to 739 mg = 220 per cent, respectively). This phenomenon is different from that observed in neonatally androgenized female rats whose ovary and uterus grow less sensitive to sex hormones [9, 19, 20, 21, 26]. We cannot, therefore, agree with MORRISON and JOHNSON [18], who have suggested that in the male rat neonatal androgen treatment *increases* the sensitivity of the accessory genital glands.

Our observations, as outlined in the foregoing, point to a reduced hypophyseal GTH production.

There are convincing data to show [11, 12, 24] that parabiosis is the best method for the determination of hypophyseal GTH production (secretion and release). The actual, prolonged production of GTH is not reliably indicated either by the determination of the level of FSH or that of LH in the hypophysis and the blood [2, 17, 22, 23]. Considering that LH stimulates the production of androgens in Leydig's interstitial cells of the male rat, changes in the weight of the reproductive organs of male detector animals admit, in the main, of inferences only to changes in LH synthesis [10, 11]. Hormones produced by the own hypophysis of detector animals undoubtedly introduce a factor of uncertainty. It is, however, probable that sex steroids produced as a consequence of gonadal hyperfunction inhibit the *in situ* synthesis of hypophyseal GTH.

In one of our earlier studies [14] the detectors were female animals. We found but a slight production of FSH in androgenized rats following castration. There is, according to our present observations, no increase whatever

in the production of LH. The weight of the accessory reproductive organs of CA rats was hardly different from that of the "non-parabiotic" animals, and the increase was less than half of what was induced by parabiosis with a CC partner.

A rise in the rate of LH production may be prevented even by that the damaged structures of the hypophyseotrophic area responsible for basic secretion became unable to produce the releasing factor. Only large doses of testosterone administered on the first days of life have such damaging effect. MORRISON and JOHNSON [18] as well as BOYD and JOHNSON [3] applied testosterone on the fifth day after birth, at a time when the treatment was no longer able to prevent the increased GTH production which is provoked by castration [15]. Administration of exogenous androgen is, of course, well suited for such inhibition. The phenomenon is understandable if we accept that androgenization has a double hypothalamic effect [1, 2, 5].

Our present results support the hypothesis regarding the double central effect of neonatal androgenization:

(1) the steroid-sensitive neural elements of the anterior hypothalamus become desensitized;

(2) the structures contained in the hypophyseotrophic area, responsible for the basal secretion of GTH, suffer damage.

It may be noted that treatment with androgen affects the regulatory activity of the hypothalamus also in respect of other trophic hormones. Production of prolactin and somatotrophin of female rats is also shifted in a direction characteristic of male animals [13, 16]. We suppose that this action too occurs in the hypophyseotrophic area, where the production of the releasing and inhibiting factors becomes changed to be characteristic of male animals.

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DIE EINSTELLUNG DER ERHÖHTEN LUTEINISIERUNGSHORMON-PRODUKTION BEI KASTRIERTEN UND ANDROGENISIERTEN RATTEN

In der Arbeit werden neue Beweise für die doppelte zentrale Wirkung der im Neugeborenenalter angewendeten Testosteronbehandlung (»Androgenisierung«) erbracht. Eine am Tage nach der Geburt subcutan verabreichte große Androgendosis (500 μg) bewirkt nach den Verfassern nicht nur eine Desensibilisierung der gegenüber Sexualsteroiden sensitiven Neuronen im vorderen Hypothalamus, sondern schädigt auch die in der hypophysiotropen Zone vorhandenen Strukturen, welche die GTH-freisetzenden Faktoren produzieren. Zur Messung der Luteinisierungshormon-Produktion wurde das Parabiose-Verfahren mit männlichen Detektor-Ratten benutzt. Das Gewicht der akzessorischen Geschlechtsorgane der Partner von kastrierten Kontrolltieren hatte sich während der 12tägigen Parabiose auf mehr als das Zweifache erhöht. Die Partner von kastrierten androgenisierten Ratten zeigten hingegen keinen Unter-

schied gegenüber den intakten individualen Tieren. Diese Beobachtung weist darauf hin, daß bei am Tage nach der Geburt androgenisierten Ratten nach dem Ausschalten des feed-back-Mechanismus die LH-Produktion keine Steigerung erfährt.

ПРЕКРАЩЕНИЕ ПОВЫШЕННОЙ ПРОДУКЦИИ ЛЮТЕИНИЗИРУЮЩЕГО ГОРМОНА У КАСТРИРОВАННЫХ И АНДРОГЕНИЗОВАННЫХ КРЫС

Предоставляются новые доказательства двойного центрального действия введения тестостерона («андрогензации») в период новорожденности. По мнению авторов подкожное введение большой дозы (500 μг) андрогена в день после рождения вызывает не только десенсибилизацию нейронов, чувствительных к половым стероидам, а повреждает также структуры, продуцирующие факторы освобождения GTH, имеющиеся в гипофизеотропной зоне. Для измерения продукции лютеинизирующего гормона авторами использовался метод парабиоза с мужскими крысами-детекторами. Во время 12-дневного парабиоза вес добовочных половых мелез партнеров кастрированных контрольных животных увеличивался выше чем в два раза. В то же время партнеры кастрированных и андрогенизованных крыс не показали отклонения от невредимых единичных животных. Данное наблюдение указывает на то, что у крысы, получившей тестостерон в день после рождения, после выключения механизма обратной связи (feed back), продукция лютеинизирующего гормона не повышается.

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EFFECT OF DIFFERENT EXPERIMENTAL INTERVENTIONS ON THE AMOUNT OF ACTH IN THE MEDIAN EMINENCE OF THE DOMESTIC PIGEON

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Synopsis

It has been demonstrated by *in vitro* incubation that the median eminence of domestic pigeons shows considerable ACTH activity. On acute treatment with insulin and under the effect of water load, the amount of ACTH in the median eminence and of that in the adenohypophysis runs parallel, while after thirsting or after the administration of prednisolone or NaCl the changes are dissimilar. The ACTH activity of the median eminence became stronger 30 minutes after the administration of 8 I.U. of insulin, to decrease very considerably by one and two hours. The activity disappeared 8 hours after the administration of 30 I.U. of insulin and decreased likewise considerably after treatment with prednisolone or ACTH as also under the influence of thirsting or water load, while decrease was less marked following treatment with NaCl. Results seem to show that extrahypophyseal ACTH represents an independent central regulator of adrenal function.

Introduction

Numerous authors have found that in birds hypophysectomy induces adrenal atrophy and a significant diminution of corticoid production [1, 3, 8, 9, 10, 11, 16]. It has further been demonstrated [3, 4, 11, 16] that, while in hypophysectomized mammals the production of glucocorticoids falls to a minimum, the adrenal gland is still able to produce a considerable amount of corticosterone. It would follow from these findings that in birds the activity of the adrenal is less dependent on the hypothalamo-hypophyseal system than in mammals.

The results of certain experiments point, on the other hand, to the existence of a central regulatory apparatus which is in many respects different from what has been observed in mammals. RESKO and co-workers [16] were the first to suggest that the median eminence of chickens contains ACTH or some similar substance. They were led to this conclusion by the observation that a lesion of the median eminence was followed by a significant drop of the corticoid level in the venous blood of the adrenal gland of hypophysectomized birds. FRANKEL and co-workers [3], who failed to demonstrate the existence of extrahypophyseal ACTH in a direct way, found that hypophysectomized birds with damaged tuberal nucleus produced more corticosterone than did hypophysectomized birds. This observation supported MILLER's [9] earlier finding

that a traumatization of the median eminence following hypophysectomy induces adrenal hypertrophy. The results of both experiments admit of the conclusion that there exists some extrahypophyseal factor which promotes adrenal activity following surgical trauma.

In earlier investigations [13, 14] we succeeded in determining the ACTH content of the median eminence. The amount of extrahypophyseal ACTH was found to be considerable much more so than that contained in the adenohypophysis. Led by the supposition that extrahypophyseal ACTH plays a functional part in the central regulation of adrenal activity, we initiated experiments in order to determine its changes in cases of reduced and intensified adrenal activities. The present experiments were carried out at the same time and under the same experimental conditions as those [15] in which we determined the CRF contents of the median eminence and the ACTH contents of the adenohypophysis. It was thus possible to determine the functional relationship between hypophyseal and extrahypophyseal ACTH.

Material and method

Adult pigeons *(Columba livia domestica)* of both sexes were used. Their number totalled 102, and their average body weight was 295 g. Female rats of the "Rehbrücke" strain with body weights between 180 and 203 g served for the determination of extrahypophyseal ACTH.

The pigeons were divided into the following groups.

(1) Forty birds were treated with insulin in acute experiment. The animals were divided into four subgroups: the members of three subgroups received a single gose of 8 I.U. insulin by the intravenous route and were sacrificed 30, 60 and 120 min after the injection; the rest received a single intravenous dose of 30 I.U. and were killed 8 hours later.

(2) Nine pigeons were given daily 5 mg prednisolone in two oral doses (10 a.m. and 6 p.m.), over a period of 20 days.

(3) Ten pigeons received daily two doses of 2 I.U. ACTH (Exacthin), i.e., a total daily dose of 4 I.U., over four days, intramuscularly. The injections were administered at 10 a.m. and 6 p.m.

(4) Twelve animals received no water during 8 days.

(5) Ten pigeons received daily 40 ml of water through a gastric tube (20 ml each, at 10 a.m. and 6 p.m.) over a period of 10 days.

(6) Ten pigeons received for 20 days maize impregnated with a 4 per cent solution of NaCl.

Both the pigeons and the rats were decapitated between 11 a.m. and 2 p.m.

The median eminence of the test pigeons was homogenized in Krebs–Ringer bicarbonate; we determined the ACTH contents of the crude extract on rat adrenal slices incubated *in vitro*, on the evidence of the resulting amount of corticosterone [13].

The values given in the Results represent the amount of corticosterone produced during an hour by 29 mg (average weight) of rat adrenal, calculated for μg/100 mg/hour. The amount of corticosterone produced by the rat adrenals was determined fluorometrically according to the method described by GLICK and co-workers [5], ZENKER and BERNSTEIN [17] and ESPINER [2] as detailed in our previous communication [13].

The tissue of median eminence, used for the determinations, weighed on an average 3 mg.

Control values were of a double kind. The correct execution of *in vitro* incubation and that of the fluorometric corticosterone determinations were controlled by a continuous measurement of the basic corticosterone secretion by the rat adrenals and, as another control, we determined the ACTH content of the median eminence in intact animals.

The adrenal glands of the sacrificed pigeons were histologically examined in all experiments in order to control the effectiveness of the interventions.

Results

The amount of ACTH in the median eminence (11.6 μg), determined 30 minutes after the administration of 8 I.U. of insulin, was found to exceed the control value significantly. It dropped significantly below the control value 1 hour after the injection (8.55 μg). A further slight decrease occurred in 2 hours (8.40 μg).

The ACTH activity of the median eminence had practically disappeared 8 hours after the intravenous injection of a large dose of insulin (30 I.U.). Compared to the basic corticosterone secretion of the rat adrenal the remaining activity (6.30 μg) was insignificant.

Treatment with prednisolone considerably reduced the ACTH contents of the median eminence (6.66 μg). The deviations of the observed values were strikingly small.

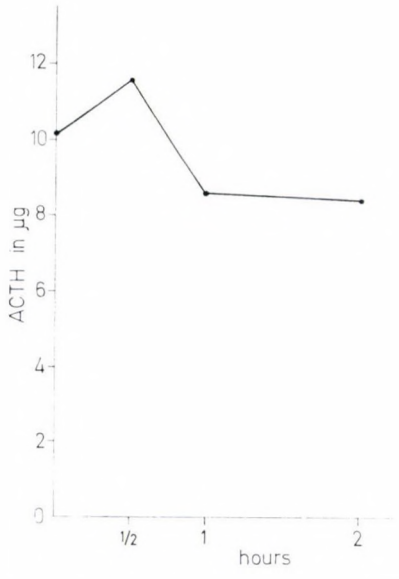

Fig. 1. Effect of a single intravenous dose of 8 I.U. insulin on the ACTH contents of the median eminence

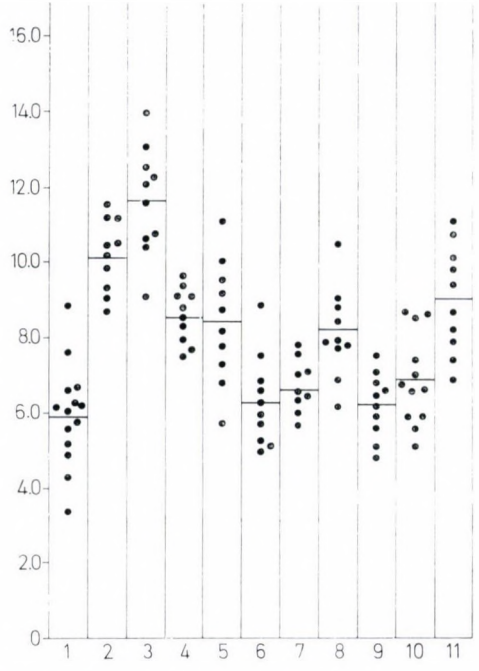

Fig. 2. Changes in the ACTH contents of the median eminence after different experimental interventions. 1 = Basic corticosterone secretion of the rat adrenal; 2 = Amount of ACTH in the median eminence of control animals; 3 = Insulin treatment — 30 min; 4 = Insulin treatment — 60 min; 5 = Insulin treatment — 120 min; 6 = Insulin treatment (30 I.U.), 8 hours; 7 = Prednisolone treatment; 8 = ACTH treatment; 9 = Water load; 10 = Thirst; 11 = NaCl treatment

Also ACTH reduced the amount of extrahypophyseal ACTH very considerably (8.20 μg); compared to the control values, the difference was significant.

Thirst induced a strong decrease in the amount of extrahypophyseal ACTH to 6.85 μg, with a likewise significant deviation from the control values.

The same phenomenon was registered after loading with water (6.19 μg). The reduction was significant in comparison to the extrahypophyseal amount of ACTH in intact pigeons, but was insignificant with reference to the basic

Table 1

Changes in the ACTH contents of the median eminence caused by different experimental interventions
Deviations are statistically significant if $t = 2.10$ and $p = 0.05$

Treatment	± value
Insulin 30 min	+2.43
Insulin 60 min	−4.16
Insulin 120 min	−2.98
Insulin 8 hours	−8.07
Prednisolone	−8.87
ACTH	−4.27
Water load	−9.79
Dehydration	−6.93
NaCl	−2.01

corticosterone secretion of the rat adrenal. The dispersion of the values was low; ACTH activity in the median eminence of the test animals practically disappeared.

Decrease in the amount of extrahypophyseal ACTH was less pronounced after treatment with NaCl (9.00 μg) (Figs 1, 2; Table 1).

Discussion

As regards the effect of a small single intravenous dose of insulin (8 I.U.) on the ACTH contents of the median eminence, an initial increase was found to have been followed by a sudden decrease. This change was quite similar to the diminution of the adenohypophyseal ACTH contents following treatment with insulin [15]. The response of the extrahypophyseal factor to insulin is, thus, of the "ACTH-type" and, therefore, of another nature than the time-conditioned change in the amount of CRF. It would follow that the extrahypophyseal factor acts as a physiological synergist of adenohypophyseal ACTH — at least after the administration of insulin.

Extrahypophyseal ACTH activity (paralleling in this respect CRF activity) dropped to zero after the intravenous injection of a large dose of insulin (30 I.U.) and also the amount of ACTH contained in the adenohypophysis showed pronounced decrement [15]. It is, therefore, evident that such radical intervention, while inducing marked adrenal hypertrophy 8 hours after the injection, resulted in the exhaustion of the hypothalamo-hypophyseal system.

Treatment with insulin or ACTH like loading with water induced parallel changes in the ACTH contents of the adenohypophysis and the median eminence, whereas changes in the respective amounts of hypophyseal and extrahypophyseal ACTH did not run together after the administration of prednisolone or NaCl and after thirsting. It was found in earlier investigations [15] that treatment with prednisolone or loading with NaCl increased the amount of ACTH in the adenohypophysis, whereas in the present experiments the hypophyseal ACTH was reduced by the same interventions.

The observation that deprivation of water causes a strong diminution in the amount of ACTH contained in the median eminence is worthy of note. Thirst has no such effect on the amount of CRF or hypophyseal ACTH [15]: it does not affect the hypothalamo-hypophyseal system. Again, thirst is known to induce adrenal hypertrophy in birds [6, 7]. Since thirst did not affect secretion in the median eminence of pigeons, we supposed that the CRF effect of the large amount of arginine-vasotocin released by the neurohypophysis, might be responsible for the hypertrophy of the adrenal gland [12]. It is, however, more probable that hypertrophy following thirsting is caused (partly at least) by ACTH released by the median eminence.

In summary, the present experiments have shown that the median eminence of pigeons contains a considerable amount of ACTH. Changes in the amount of extrahypophyseal ACTH are independent of those in the amount of hypophyseal ACTH, suggesting that extrahypophyseal ACTH acts as an independent central regulator of adrenal activity.

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DIE WIRKUNG VERSCHIEDENER EXPERIMENTELLER EINGRIFFE AUF DEN ACTH-GEHALT IN DER EMINENTIA MEDIANA DER HAUSTAUBE

Mit Hilfe von in vitro Inkubation wurde festgestellt, daß die Eminentia mediana der Haustaube über eine bedeutende ACTH-Aktivität verfügt. Unter Insulinwirkung und Wasserbelastung verändert sich der ACTH-Gehalt der Eminentia mediana parallel dem ACTH-Gehalt der Adenohypophyse, während sich nach Prednisolongaben und NaCl-Belastung dieser Effekt nicht einstellt. Diese Befunde weisen darauf hin, daß das extrahypophyseale ACTH einen selbständigen zentralen Regulationsfaktor der Nebennierenfunktion darstellt.

ДЕЙСТВИЕ РАЗЛИЧНЫХ ЭКСПЕРИМЕНТАЛЬНЫХ ВМЕШАТЕЛЬСТВ НА СОДЕРЖАНИЕ АКТГ В EMINENTIA MEDIANA ДОМАШНЕГО ГОЛУБЯ

Методом инкубации in vitro было установлено, что eminentia mediana домашнего голубя обладает значительной активностью АКТГ. Под влиянием острото воздействия инсулином или нагрузки водой содержание АКТГ изменяется параллельно концентрации АКТГ в аденогипофизе, тогда как под влиянием преднизолона, жажды и нагрузки поваренной солью этого эффекта не наблюдается. Результаты указывают на то, что внегипофизарное содержание АКТГ представляет самостоятельный центральный фактор регулирования функции надпочечников.

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COMPARATIVE HISTOCHEMISTRY AND ELECTRON MICROSCOPY OF THE EOSINOPHIL LEUCOCYTES OF VERTEBRATES

I. A STUDY OF AVIAN, REPTILE, AMPHIBIAN AND FISH LEUCOCYTES

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Synopsis

Leucocytes of the blood and haemopoietic tissues of lower vertebrates (birds, reptiles, amphibia and fishes) were studied by light and electron microscopic methods. The granules of avian, reptile and amphibian eosinophil leucocytes showed high isoelectric point (alkaline erythrosin), anisotropic staining by Congo red or Sirius red F3B, fluorescence by thioflavine S and positive neutral and alkaline benzidineperoxidase reaction. In fishes (carp, river bleak and tench) two types of leucocytes were recognized. None of their granules exhibited high isoelectric point, Congo red or Sirius red F3B anisotropy or thioflavine S fluorescence, but in one leucocytic cell line the granules gave a positive neutral benzidine peroxidase reaction (cell type 2); in the other both the neutral and the alkaline reactions were negative (cell type 1). The latter cells showed a high number of small granules and a frequent occurrence of "envelope-limited sheets of chromatin" and were classified as neutrophil leucocytes. The benzidine-peroxidase positive granules of the former cells were fewer and relatively large. It is believed that these cells represent "eosinophil" leucocytes. In all species studied the matrix of the leucocytic granules frequently showed a granular or fibrillar transformation, reminding of a similar change of the rat and human eosinophil granules. The matrix transformation is regarded as a sign of enhanced functional (enzymatic) activity. In the leucocyte granules of reptiles and amphibia indentations or excavation-like holes were often seen, representing probably sites of interaction between the granules and the cytoplasm.

Introduction

Eosinophil leucocytes are regular elements of the blood and bone marrow in mammals and have been reported to be present also in lower vertebrates (birds, reptiles, amphibia, fishes and cyclostomes) [2, 10, 11, 13, 14, 17—19, 27, 32, 37, 45]. Due to the size, high isoelectric point (acidophil staining) and characteristic submicroscopic structure of their specfic granules the eosinophil leucocytes of some rodents (hamster, mouse, rat and guinea-pig) and man are conspicuous both in the light and the electron microscope.

The high isoelectric point of the eosinophil granules seems to be related to a baisic protein not separable from their peroxidase-type enzyme [5]. A further characteristic feature of the eosinophils is their benzidine peroxidase reaction, which is more intense in the eosinophils than in the neutrophil granules [35, 42]. Besides this enzyme, alkaline and acid phosphatase and ATP-ase have been demonstrated in the eosinophil granules [21, 46, 54, 56]. The presence of these hydrolytic enzymes and basic proteins renders the eosinophil granules similar to lysosomes [55].

In the electron microscope the eosinophil granules are built up of an outer, membrane-bound, slightly osmiophil matrix (externum) and an inner, crystal-like, strongly osmiophil structure, called crystalloid (internum) [4, 38, 48]. So far acid phosphatase, ATP-ase and peroxidase have been demonstrated in the matrix by electron histochemical methods [21, 30, 36, 47, 56].

Cell kinetic studies have shown that in the rat the eosinophils, after being discharged from the bone marrow, spend only a limited time in the blood (90 minutes to 24 hours) and thereupon enter the tissues, the site of their main activity [7, 12, 20]. According to recent observations large numbers of eosinophil leucocytes break down under physiological condi-

tions in the intestinal wall, peribronchial tissues and spleen [51]. The number of eosinophils in the bone marrow reflects the rate of their production, while in the blood their number depends on their release from the bone marrow and on the number of cells leaving the blood for the tissues; the latter seems to influence not only the rate of discharge, but also the time of differentiation and maturation of the bone marrow eosinophils [12].

Tissue eosinophilia is usually indicative of a pathological reaction to foreign proteins entering the tissues. Although the physiological and pathophysiological role of the eosinophils is not clearly understood, their capacity of ingesting of immunologically active proteins, antigens, or antigen-antibody complexes seems to have been established [33, 39, 43, 49].

When trying to compare the eosinophil leucocytes of different species it seems necessary to define the cells which may be considered eosinophils on the basis of morphological criteria. The eosinophil leucocytes of some rodents (hamster, mouse, rat and guinea-pig), rabbit and man show a number of characteristic features.

(1) When stained with the Giemsa-solution the eosinophil granules are orange-yellow in colour after methanol and bluish-black after acetone-fixation [30].

(2) High isoelectric point of the specific granules due to a basic protein which selectively binds acid dyes in alkaline media by ionic-type linkages (eosin, erythrosin, acid fuchsin, phloxine, aniline blue, Biebrich scarlet, Cochineal red) [9, 15, 50].

(3) Selective anisotropic staining and selective fluorochroming by substantive dyes and fluorochromes, as Congo red or Sirius red F3B and thioflavine S. The substantive dyes are bound to the granules by nonionic-type linkages [28, 29].

(4) Positive benzidine peroxidase reaction, selective for the eosinophils if performed in an alkaline buffer, at pH 11—12 (alkaline benzidine peroxidase reaction [35]). When incubated at a less alkaline medium (pH 7—8, neutral benzidine peroxidase reaction) both the eosinophil and the neutrophil granules will react. The enzyme-reaction is blocked at an intermediate stage by phosphotungstic acid pretreatment [30].

(5) At the electron microscopic level the specific granules consist of matrix and crystalloid, surrounded by a single membrane [4, 38, 48].

(6) Affinity of the granule matrix to phosphotungstic acid [16, 22, 24, 30].

It may be seen that it is the specific granules of the eosinophils that all these features are typical of. Other cellular details is these species are similar to those of the neutrophils. Therefore, in the species studied our attention was primarily focussed on the granules of the leucocytes.

The avian, reptile and amphibian eosinophils meet all the above criteria, except that the granules of these species do not possess crystalloids. In the leucocytes of the fishes studied none of the granules were of a high isoelectric point; due to this circumstance it was difficult to decide on the cell line to be regarded as "eosinophil". Furthermore it should be mentioned that in birds and reptiles and in the rabbit the granules of the cells corresponding to human

and rat neutrophils, similarly to the eosinophils, show a high isoelectric point (acidophil staining in alkaline media); that might account for their being called heterophils [27], or pseudoeosinophils.

The comparative studies to be described seemed promising from several aspects: (1) They might furnish some evidence in phylogenetical questions. (2) The relationship of certain histochemical reactions might elucidate the identity or non-identity of the factors responsible for the underlying reactions, e.g., in fishes none of the leucocytes show high acidophilia, but one cell line is peroxidase active; thus peroxidase activity and high isoelectric point are not intrinsically related. (3) The presence or absence of a histochemical reaction enables a certain submicroscopic structure to be related to a certain histochemical reaction; e.g., the absence of crystalloids from the avian, reptile and amphibian granules and the positivity of the peroxidase reaction and acidophilia may suggest that the crystalloid is not necessarily related to the peroxidase reaction and acidophilia. (4) The finding of a well-developed system of eosinophil leucocytes may throw some light on the immunological potentialities of the species [11]. (5) An alteration in the granules of the eosinophils, called "granular-vesicular matrix transiformation", has been described [31; this change of the granule-matrix seemed to be related with enhanced functional (enzymatic) activity. The frequent occurrence of similar changes in the granules of lower vertebrates, as observed in the present study, might contribute to a better understanding of the functional morphology of the granules.

Material and method

Blood and haemopoietic tissues of the following animals were studied.

(1) *Birds:* chicken *(Gallus domesticus)* and pigeon *(Columba domestica)*; bone marrow and blood.

(2) *Reptiles:* lizard *(Lacerta agilis)* and turtle *(Emys orbicularis)*; liver, spleen and blood.

(3) *Amphibia:* frog *(Rana esculenta)* and tritons *(Triturus cristatus* and *Triturus vulgaris)*; bone marrow, liver, spleen and blood.

(4) *Fishes:* carp *(Cyprinus carpio)*, river bleak *(Carassius carassius)* and tench *(Tinca tinca)*; kidney and blood.

Air-dried smears of blood, bone marrow and other organs were used either unfixed or fixed in absolute methanol, absolute acetone or 4 per cent formaldehyde (in 0.15 *M* phosphate-buffer, pH 7.2, containing 7.5 per cent sucrose) at room temperature for 5 minutes. For light microscopy bone marrow and organ tissues were fixed in 4 per cent formaldehyde (in 0.15 *M* phosphate-buffer, pH 7.2, containing 7.5 per cent sucrose) at 4 °C for 12—24 hours, rinsed in formaldehyde free buffer and, after dehydration in acetone, embedded in paraffin.

Staining methods and enzyme-reaction: (a) alkaline erythrosin (Gurr), 0.1 per cent (in citric acid–boric acid–phosphoric acid–sodium hydroxide-buffer, pH 8—12), for 3—5 minutes at room temperature; (b) Congo red (Riedel), 0.1 per cent, Sirius red F3B (Bayer), 0.1 per cent and thioflavine S (Harleco), 0.1 per cent, for 5—60 minutes at room temperature. (c) Benzidineperoxidase reaction: acetone-fixed smears, formaldehyde-fixed frozen sections, or small tissue blocks (diameter 1—2 mm) were kept in a buffered solution (citric acid–boric acid–phosphoric acid–sodium hydroxide, pH 7—12) saturated with benzidine containing 0.015 per cent hydrogen peroxide, added immediately before use; incubation time, 5—20 minutes at room temperature. Neutral benzidineperoxidase reaction: incubation at pH 7; alkaline benzidineperoxidase reaction: incubation at pH 11—12. After incubation smears

were washed in distilled water and dried; frozen sections were first rinsed in distilled water, then stained with a 1 per cent toluidine blue solution (in Michaelis buffer, pH 4.0) for 5—10 minutes, blotted with filter paper, rinsed with a 2 per cent potassium ferricyanate solution and mounted in Gum arabic containing 0.2 per cent potassium ferricyanate [41]. Benzidineperoxidase-reacted tissue blocks were dehydrated in ethanol and embedded in paraffin or Araldite (Ciba, Basel) (see below).

Polarization microscopy: Type "Ortholux–II" polarization microscope (Leitz, Wetzlar) with a rotating mica compensator.

Fluorescence microscopy: "Zetopan with Binolux" fluorescence microscope (Reichert, Vienna) with an "UV-Sperrfilter".

Electron microscopy. Small tissue blocks of 1—2 mm diameter were fixed immediately upon removal in osmium tetroxide, 1.0 per cent (in 0.15 *M* phosphate-buffer, pH 7.2 with 7.5 per cent sucrose), at 4 °C for 60—90 minutes; in formaldehyde 4 per cent (in 0.15 *M* phosphate-buffer, pH 7.2 with 7.5 per cent sucrose), at 4 °C for 30—60 minutes; in glutaraldehyde (Fluka), 2.5—5 per cent (in 0.2 *M* cacodylate-buffer, pH 7.2), at 4 °C for 30—60 minutes. After fixation, tissues were washed in the fixative-free buffer solution, dehydrated in ethanol and embedded in Araldite (Ciba) or Maraglas (Polyscience). Formaldehyde-fixed tissues were stained, in some cases, with phosphotungstic acid, 0.1—1.0 per cent, in 70 per cent ethanol during dehydration, for 30—60 minutes.

An Ultrotome ultramicrotome (Type 4801 A, LKB-Produkter, Stockholm) with glass knives was used. The 0.5—1.5 μ thick sections were stained with haematoxylin-eosin; the sections mounted on microscope slides were dried under infrared light and treated for one hour with a 1 per cent solution of periodic acid (in distilled water), stained with Ehrlich's haematoxylin for 12 hours, then the sections were kept in running tap water for 15—30 minutes, shortly differentiated in acid alcohol (1 ml of cc. hydrochloric acid in 96 per cent ethanol), followed by water, 30 minutes. Counterstain: eosin, 0.5 per cent (spiritlös., Grübler) in 50 per cent ethanol for 60—120 minutes, dehydrated in absolute ethanol, blotted with filter paper, cleared in xylol and mounted in Canada balsam. The ultrathin sections were contrasted on copper grids with a saturated solution of uranyl acetate in 70 per cent ethanol for 10—30 seconds, and with lead citrate [53] for 120 seconds. The electron micrographs were taken on a Tesla BS 242 D (Tesla, Brno) and on a JEM-6A (JEOL Co., Ltd., Tokyo) electron microscope.*

Observations

In Table 1 the light and the electron microscopic observations are summarized.

In Giemsa-stained blood and bone-marrow smears from *birds* (chicken and pigeon) the eosinophil leucocytes can be easily recognized; after methanol-fixation the granules are of orange-yellow, after acetone of brownish-black colour. Both heterophil and eosinophil granules are large, the mature heterophil granules rod-like or oval (Fig. 2), the immature ones, just like the somewhat smaller eosinophil granules, round or slightly oval. Absolute methanol or acetone will dissolve the mature and some of the immature heterophil granules, but not the eosinophils. When unfixed or formol-fixed smears are stained with the Giemsa solution, no or only slight dissolution of the heterophil granules occurs. Both heterophil and eosinophil granules show high isoelectric points (alkaline erythrosin), Congo red or Sirius red F3B anisotropy and selective thioflavine S fluorochroming; thus, eosinophils cannot be differentiated from the heterophils by these methods. The benzidineperoxidase reactions, both the neutral and the alkaline, however, are positive only in the eosinophil granules (Fig. 3). If the smears are treated, before incubation, for 1 to 5 minutes with a 0.1 to 1 per cent solution of phosphotungstic acid, the benzidineperoxidase reaction is blocked at an intermediate stage, i.e., after the development of the blue colour ("benzidine-blue") the reaction does not proceed to its final stage (formation of "benzidine-brown"). In sections of bone marrow the erythropoietic cells are separated from the leucocytes, the former being in the medullary sinuses, the latter in the extravascular spaces (Fig. 1).

* Our thanks are due to the Department of Anatomy, Medical University, Pécs and to the Department of Histology, Medical University, Budapest, for the use of their electron microscopes.

Table 1

Light and electron microscopic observations on the granules of the neutrophil (heterophil) and eosinophil leucocytes of the species studied

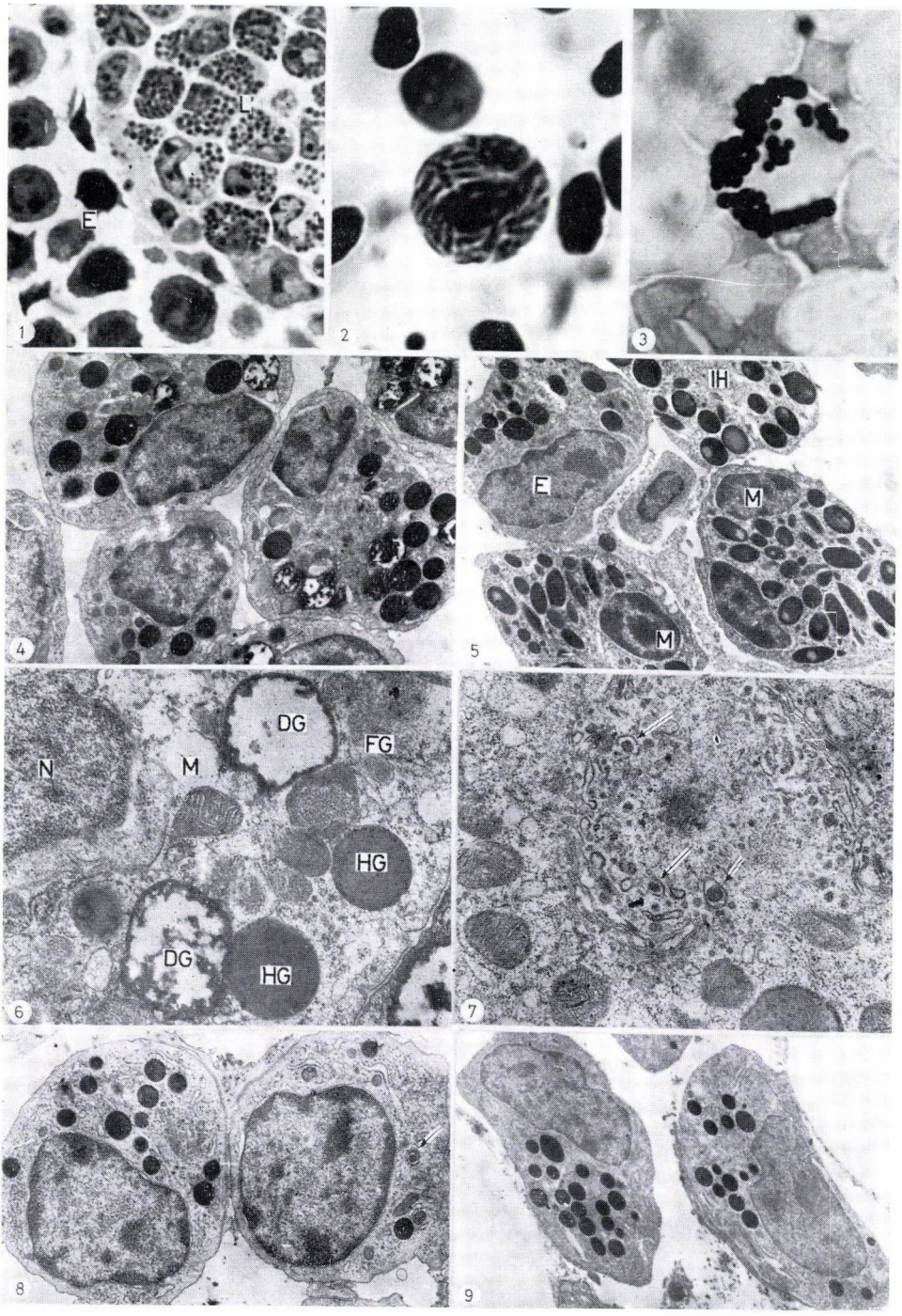
Species	Rat, mouse, man		Rabbit		Birds		Reptiles		Amphibia		Fishes	
Type of leucocyte	N[b]	E[c]	H[d]	E	H	E	H	E	N[e]	E	N (type 1)	"E" (type 2)
Benzidine peroxidase reaction, neutral	+	+	+	+	−	+	−	+	−(?)+		−	+
alkaline	−	+	−	+	−	+	−	+	−(?)+		−	−
High isoelectric point	−	+	+	+	+	+	+	+	+		−	−
Anisotropy with Congo red or Sirius red F3B[a]	−	+	+	+	+	+	+	+	+		−	−
Fluorescence with thioflavine S[a]	−	+	+	+	+	+	+	+	+		−	−
Electron microscopy: presence of crystalloids or cores	±	+	−	+	±[f]	−	−	−	±	±[g]	±	±
Granular, vesicular or fibrillar matrix transformation	−	+	−	+	+	−	+	+	−	+	+	+

[a]for details, see Methods
[b]N = neutrophil, [c]E = eosinophil and [d]H = heterophil leucocytes
[e]neutrophil granules of amphibia are beyond light-microscopic visibility
[f]only after dissolution of the matrix
[g]after phosphotungstic acid (see Fig. 26)

In the electron micrographs of osmium tetroxide-fixed bone marrow, due to their large number and the partial dissolution of their round granules, the immature heterophils (promyelocytes, myelocytes) are the most conspicuous. In the more mature forms these round granules (diameter, 0.4—1.0 μ) become gradually elongated and take up an oval or rod-like form (diameters, 0.1—0.5 × 0.7—2.0 μ). Mature and immature heterophil granules may show an electron-dense central core, especially apparent, when other parts of the granule matrix are dissolved (Fig. 4). In a few other granules of the immature cells a distinct fibrillary structure can be discovered (Fig. 6). The heterophil granules seem to originate at the Golgi region of the cells, appearing as small, gradually increasing, electron-dense droplets in the vesicles (Fig. 7).

The granules in the eosinophil leucocytes are smaller in number than those in the heterophils (Fig. 8), they are round or oval (diameter, 0.3—0.8 μ) and of homogeneous electron density; they do not show central cores and are surrounded by a single membrane. Although we did not observe any eosinophil promyelocyte in the full stage of granule formation, the appearance of small electron-dense droplets in the Golgi vesicles is indicative of their origin from these structures.

In aldehyde-fixed and phosphotungstic acid-stained marrow the granules of both the heterophils and eosinophils are electron-dense; some of the heterophil granules (both the round and rod-like forms) show a less electron-dense central patch of indistinct outline (Figs 5 and 9).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In *reptiles* (lizard and turtle) the islands of bone marrow, enclosed in a dense network of spongious bone tissue, were too small to be processed for embedding. The liver and the spleen, on the other hand, were harbouring immature and mature leucocytes, sufficient in number for both light and electron microscope studies.

The mature and immature heterophil and the eosinophil granules showed the same characteristics as the corresponding cells of birds: round immature and rod-like mature heterophil and round or slightly oval eosinophil granules (Fig. 10), high isoelectric point (alkaline erythrosin), anisotropy with Congo red or Sirius red F3B and fluorescence with thioflavine S. The benzidineperoxidase reaction, both the neutral and the alkaline, were found to be positive only in the eosinophil granules. In contrast to the bird leucocytes, the immature heterophil cells of the reptiles did not show dissolution of their granules; in the eosinophils the centre of the granules appeared more transparent than the periphery.

In the electron microscope the immature heterophil granules are round (diameter, 0.5—1.4 μ), and with cellular maturation become more and more elongated (Fig. 11). The most apparent feature of the immature heterophil cells is the great number of fine vesicles, possibly Golgi vesicles, present throughout the cytoplasm. Many of the immature heterophil granules show small peripheral electron-lucent, distinctly outlined excavations or holes (Figs 11—17). The peripheral limiting sheet of these structures is sometimes disrupted and protruding into the cytoplasm (Figs 16—17). The mature heterophil granules are rod-like (diameter, 0.4—0.8 $\times$ 0.7—1.8 μ); these granules are not as sharply outlined as the immature ones, and show a ragged appearance (Fig. 11). The cytoplasm of the mature heterophils is more electron-dense than that of the immature cells, and the number of fine vesicles is greatly reduced; these observations might indicate early cellular decay. In the spleen of reptiles a large number of immature heterophil and eosinophil cells in different stages of cellular breakdown can be found. In these cells the matrix of the granules may show variable forms of granular, vesicular or fibrillar transformation (Figs 18—19).

The eosinophil granules are round or slightly oval (diameter, 0.5—1.2 μ); the granule matrix appears homogeneously electron-dense, or partly dissolved, thus, a finely granular and fibrillar structure becomes apparent. These granules do not show central cores and are surrounded by single membrane. The eosinophil granules originate, like in the birds, in the Golgi vesicles as small electron-dense droplets.

In the *amphibia* the haematopoietic tissue examined was either the bone marrow (frog) or the liver capsule (tritons). In contrast to birds, there are no rod-like heterophil granules, the granules of the cells corresponding to the heterophil leucocytes (neutrophils) are very

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Fig. 1. Chicken bone marrow (OsO_4–Araldite, 1 μ thick section, haematoxylin-eosin). Extravascular leucocytes (L) and intrasinusoidal erythropoietic elements (E). Appr. $\times$450

Fig. 2. Heterophil leucocyte, chicken blood (methanol–Giemsa). The cell is filled with elongated rod-like granules. Appr. $\times$950

Fig. 3. Eosinophil leucocyte, pigeon bone marrow (alkaline benzidine peroxidase reaction). The round or slightly oval granules give an intense reaction. Appr. $\times$950

Fig. 4. Heterophil myelocytes, chicken bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). Large, round, homogeneously electron-dense granules, in some the matrix is dissolved. Appr. $\times$3,500

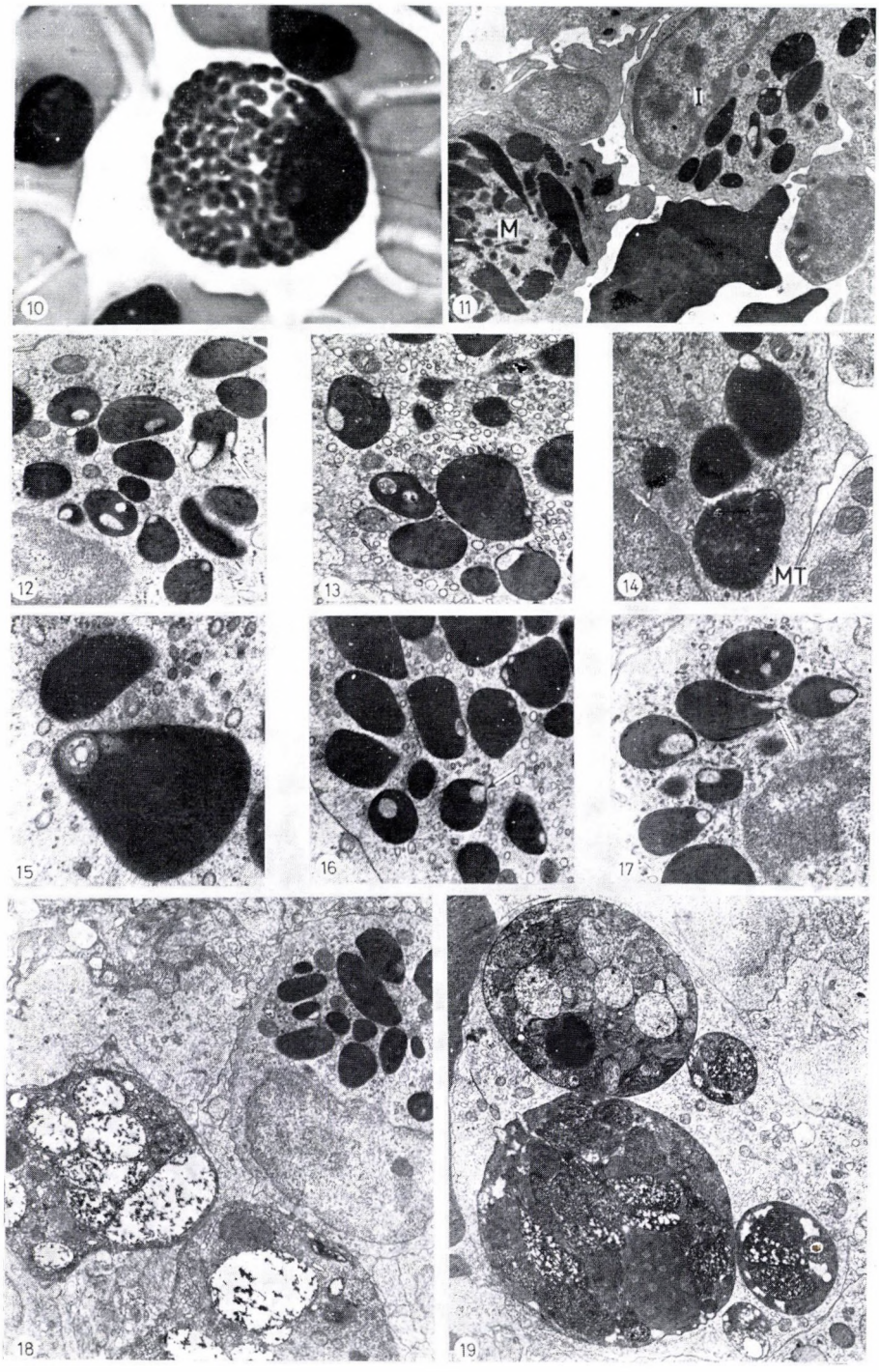
Fig. 5. Mature (M) and immature (IH) heterophil and an eosinophil (E) leucocyte, pigeon bone marrow (formol-phosphotungstic acid–Araldite). The centre of both the mature and immature heterophil granules is less electron-dense than their periphery. The eosinophil granules seem to be homogeneous. Appr. $\times$3,500

Fig. 6. Heterophil myelocyte, pigeon bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). N = nucleus, M = mitochondrium, HG = homogeneous granules, DG = granules with dissolved matrix, FG = fibrillar granule matrix. Appr. $\times$9,000

Fig. 7. Heterophil myelocyte, chicken bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). Formation of granules in the Golgi vesicles (arrows). Appr. $\times$9,000

Fig. 8. Eosinophil myelocytes, chicken bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). A few round, homogeneously electron-dense granules. Arrow points to granule formation in a Golgi vesicle. Slightly dilated ergastoplasmatic cisternae. Appr. $\times$3,500

Fig. 9. Eosinophil metamyelocytes, chicken bone marrow (formol–phosphotungstic acid–Araldite). Relatively few round or slightly oval granules, homogeneously electron dense. Appr. $\times$3,500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small (beyond the light microscopic visibility); the eosinophil granules are large, thus, the two cell lines can be easily differentiated.

In Giemsa-stained smears from blood, bone marrow, liver and spleen the eosinophils appear as large cells with round or slightly oval granules. The neutrophil leucocytes are characterized by 3—5 nuclear lobes and by the lack of definite granules. The eosinophil granules show high isoelectric points (alkaline erythrosin), selective anisotropy with Congo red or Sirius red F3B, selective fluorescence with thioflavine S and positivity with both the neutral and the alkaline benzidine peroxidase reaction. It should be noted that in the neutrophils the benzidine peroxidase reaction is negative: there is no trace of any brown discolouration in their cytoplasm. In the eosinophil leucocytes, besides the large forms, one might discover another population of granules; these are small elements giving the same reactions as the large granules. They can be detected only if the cells are spread out enough, so that the granules are individually discernible; in 0.5—1.5 μ thick sections both large and small granules are visible (Figs 20—21).

When examined under the electron microscope, the neutrophil leucocytes possess a mixed population of granules (Fig. 23); a smaller type (diameter, 0.1—0.3 μ), being more numerous than the larger granules, which are elongated (0.1—0.3 × 0.5—0.9 μ) and more electron-dense. In these latter, a crystalloid-like inner structure may be present. The smaller granules seem to develop in the Golgi vesicles, which may occur even in mature cells. The origin of the larger granules was not established. In immature cells the larger granules predominate, which, although smaller, remind of the rod-like heterophils of birds and reptiles (Fig. 22). A few of the amphibian neutrophils show peculiar chromatin bridges ("envelope-limited sheets of chromatin" [13]) (Fig. 23).

In the cytoplasm of the eosinophil leucocytes both large and small granules can be observed. The large granules (diameter, 0.6—1.4 μ) are generally homogeneous and surrounded by a single membrane; the small granules, although more electron-dense, show the same features (Fig. 24). The small granules seem to originate in the Golgi vesicles. Like in the large granules of immature heterophils of the reptiles, the amphibian eosinophil granules may also show sharply outlined peripheral excavations or indentations, communicating with the cytoplasm (Figs 25—26). In a few eosinophil leucocytes (mature or immature) the granules may appear as irregularly outlined bodies with varying degree of structural disorganization. These changes (decrease in the electron density of the matrix, the appearance of a granular, vesicular, or fibrillar matrix structure), like those of the heterophils of the reptiles, are suggestive of an early cellular breakdown.

Phosphotungstic acid increases the density of both the neutrophil and eosinophil granules (Fig. 26).

In the *fishes* studied (carp, river bleak and tench) the main site of haemopoiesis is the kidney (Fig. 27). In acetone- or methanol-fixed and Giemsa-stained smears of the blood or kidney two distinct cell lines of leucocytes can be immediately recognized. They are of similar size, their nuclei are round, oval or kidney-shaped and located at the periphery of the cells. In one cell line (type 1) a slight degree of nuclear segmentation may occur, in the other (type 2) the nuclei undergo gradual pyknosis. In the blood all elements of type 2 possess pyknotic nuclei. The cytoplasm of *type 1 cells* is vacuolated; i.e., the outlines of the granules are visible. *Type 2 cells* are filled with granules the centre of which is red, the periphery appears as unstained (Fig. 28). They do not show high isoelectric point (alkaline erythrosin), anisotropy with Congo red or Sirius red F3B and fluorescence with thioflavine S. The benzidine peroxidase reaction, performed at pH 8, is positive, at pH 12 (alkaline benzidineperoxidase reaction), however,

Fig. 10. Eosinophil leucocyte, turtle blood (methanol-Giemsa). Cytoplasm filled with round or slightly oval specific granules. Appr. ×950

Fig. 11. An immature (I) and a mature (M) heterophil leucocyte, turtle spleen (OsO_4–Maraglas, uranyl acetate–lead citrate). The granules of the immature cell are oval and sharply outlined, in the other cell the granules are more elongated and show a ragged appearance. Appr. ×3,500

Figs 12—17. Granules of immature heterophil and eosinophil leucocytes, turtle spleen (OsO_4–Maraglas, uranyl acetate–lead citrate). Excavation-like holes or indentation in the matrix of the granules. Arrows point to communications between the matrix and the cytoplasm. In Fig. 14 granular matrix transformation. Appr. ×9,000 except Fig. 15: ×14,000

Figs 18—19. Phagocyted and intact leucocytes, turtle spleen (OsO_4–Maraglas, uranyl acetate–lead citrate). The granule matrix in the phagocyted cells shows various forms of granular and fibrillar transformation. Appr. ×5,000

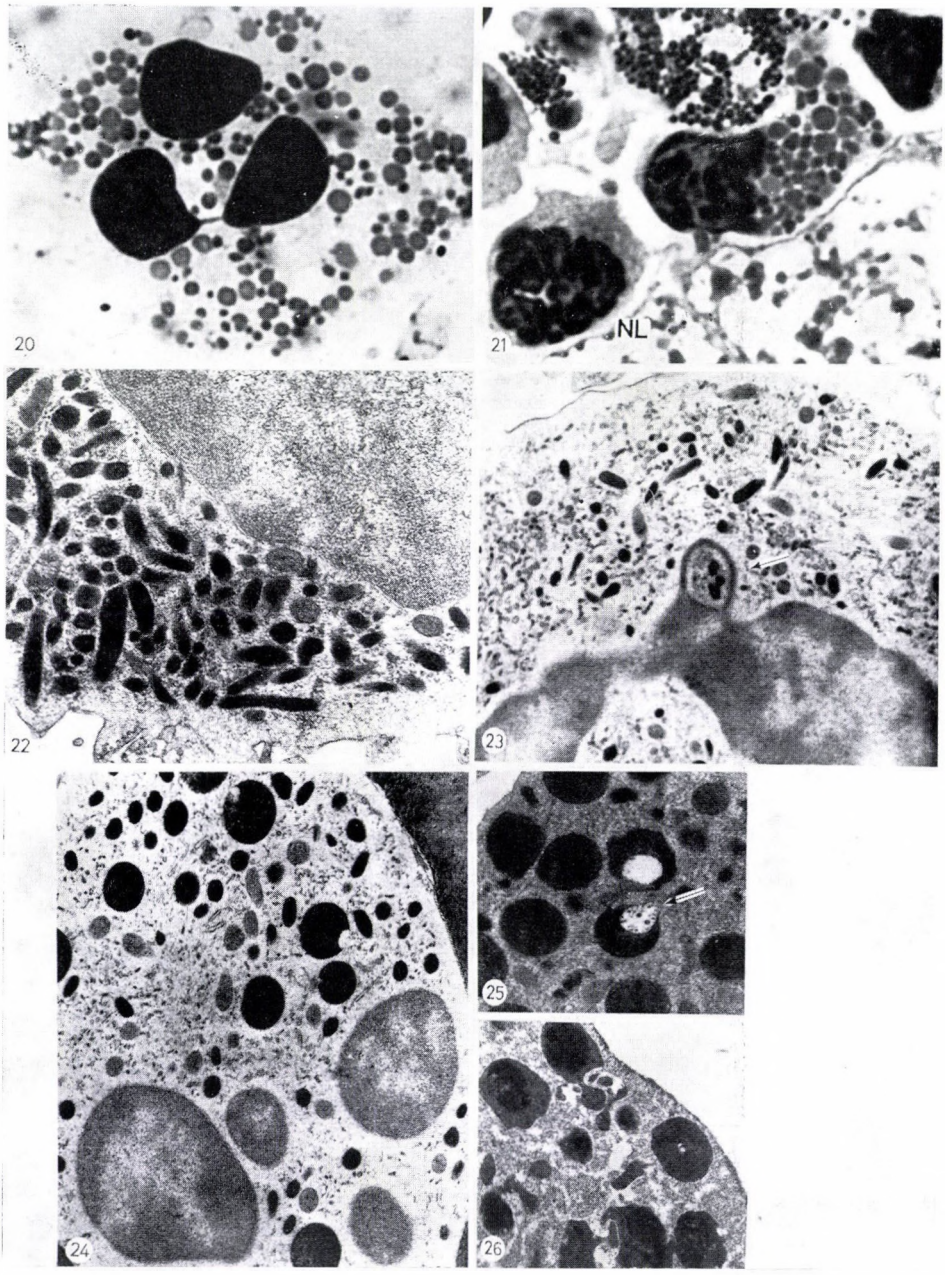

Fig. 20. Eosinophil leucocyte, frog bone marrow (methanol–Giemsa). Large and small round granules. Appr. ×1,200

Fig. 21. Eosinophil leucocyte, triton liver (OsO_4–Araldite, 1 μ thick section, Giemsa). Large and small round granules in the eosinophil leucocyte; a neutrophil leucocyte (NL) without discernible granules Appr. ×1,200

Fig. 22. Immature neutrophil leucocyte, frog bone marrow (OsO_4–Araldite, uranyl acetate–

only a very weak reaction can be observed (Fig. 29). The reaction is invariably blocked by pretreatment with phosphotungstic acid. Type 1 cells are more numerous in the carp and river bleak and type 2 cells in the tench. In Giemsa-stained 0.5—1.5 μ thick sections of osmium-tetroxide-fixed kidney the two cell lines can be easily differentiated, also cells of type 1 possessing granules; these are smaller and more numerous than the granules of cell type 2.

In the electron microscope type 1 cells show at least two types of granules. The larger ones are oval (diameter, 0.1—0.4 × 0.5—1.3 μ) and contain crystalloids, enclosed in an electron-lucent envelope; the matrix of the granules is electron-dense and exhibits a definite periodicity in the form of dark and light bands of about 100 Å width (Figs 30—31). Another type of granule is smaller (diameter, 0.1—0.4 μ) and consists of an electron-dense matrix surrounded by a single membrane. The larger granules of cell type 1 originate at the periphery of the Golgi region of cells; mature forms of these granules may appear in a radial orientation around the Golgi region. In the nuclei of type 1 cells we frequently observed nuclear bridges known as "envelope-limited sheets of chromatin" [13] (Fig. 32). Type 2 cells show large, round or oval granules (diameter, 0.8—1.4 μ), which are homogeneous or partly granular-fibrillar, occasionally with a central electron-dense core (Fig. 33). The Golgi region of these cells is well-developed and a large number of fine vesicles may be observed, scattered throughout the cytoplasm. The granules seem to originate in the Golgi vesicles.

Phosphotungstic acid increases the contrast of the granule matrix of both cell types, but does not stain the crystalloids of the large granules in cell type 1. The "envelope-limited sheets of chromatin" are strongly stained by phosphotungstic acid (Fig. 32).

Discussion

In the present comparative study of the leucocytes of lower vertebrates it seemed important to homologize the corresponding elements. On the basis of their histochemical reactions and electron microscopic features the heterophil (neutrophil leucocytes) of birds, reptiles and amphibia could easily be differentiated from the eosinophils (Table 1). It should be reiterated that, because of the strong acidophil staining of their granules the cells corresponding to the neutrophils of mammals in birds and reptiles — and also in the rabbit — are called heterophils or pseudoeosinophils [27]. In the fishes studied we did not find leucocytes having granules of high isoelectric point, i.e., showing intense acidophil staining in alkaline medium. It should be mentioned that the recent comparative studies of ANDREW [2] and of FEY [19] are contradictory in the classification of fish leucocytes. Figs 5—8 in the monograph of the former author and Fig. 4 in the paper of the latter author represent a leucocyte of the same type (called by us type 1 cell), named eosinophil by ANDREW [2] and neutrophil by FEY [19]. What speaks in favour of considering the leuco-

lead citrate). Uniform population of elongated, homogeneously electron-dense granules. Appr. ×8,000

Fig. 23. Mature neutrophil granulocyte, frog bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). Granules of various types; at arrow "envelope-limited sheet of chromatin", enclosing cytoplasmic structures. Appr. ×6,000

Figs 24—25. Eosinophil leucocyte, frog bone marrow (OsO_4–Araldite, uranyl acetate–lead citrate). Smaller and larger granules, the latter with indentations; in one of them (Fig. 25) fine granular-vesicular structures are visible (arrow). Appr. ×6,500 and ×7,000

Fig. 26. Eosinophil leucocyte granules, frog bone marrow (formol–phosphotungstic acid–Araldite). Irregularly outlined small and large granules, in some an electron-dense core. Appr. ×7,500

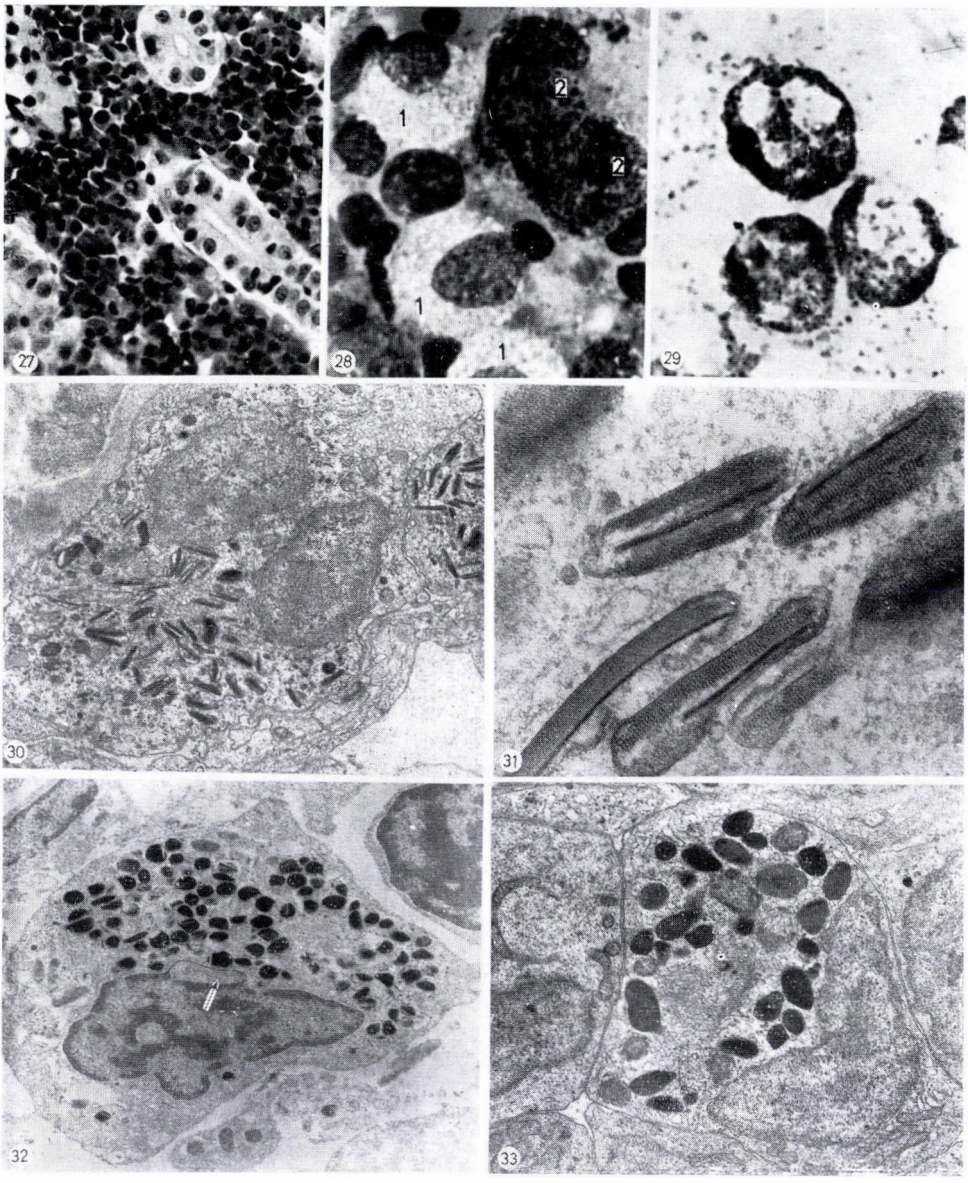

Fig. 27. Carp kidney (formol–paraffin, haematoxylin-eosin). Between the kidney tubules haemopoietic elements. Appr. ×140

Fig. 28. Kidney smear, river bleak (methanol–Giemsa). Leucocytes with weakly stained cytoplasm and without sharply outlined granules: type 1 cells (1) and cells with intensely stained granules: type 2 cells (2). Appr. ×900

Fig. 29. Kidney smear, carp (ethanol–neutral benzidine peroxidase reaction). Type 2 cells show intense reaction. Appr. ×850

Figs 30— 31. Type 1 cell, carp kidney (OsO_4–Maraglas, uranyl acetate–lead citrate). Around the Golgi region elongated granules with crystalloids. The granule matrix shows a definite periodic structure. Appr. ×5,000 and ×44,000

cytes of type 2 as "eosinophils" is our observation of their large granules of round or slightly oval shape, reminding of the eosinophil granules of higher vertebrates (reptiles and amphibia) and their positive benzidine peroxidase reaction. From the phylogenetic point of view it seems of interest that in the leucocytic granules of lower vertebrates the benzidine peroxidase reaction, first the alkaline, then the neutral, gradually becomes negative, as we go earlier on the phylogenetic scale. The benzidine peroxidase reaction, as described by UNDRITZ and co-workers [51], was found to be useful in the phylogenetic studies of the eosinophil leucocytes of different mammals. The presence of crystalloids in the granules of type 1 leucocytes of fishes does not exclude their being neutrophils; in amphibian and in human neutrophil granules there is a type which has a crystalloid-like electron-dense inner core [6]. An accidental observation of CLAWSON and co-workers [11] on the paddlefish *(Polyodon spathula)*, a fish more primitive than those used in our studies, clearly indicates that the eosinophils of lower vertebrates have no crystalloid-like structure. Another circumstance in favour of regarding type 1 cells as neutrophils is the occurrence of peculiar chromatin bridges in these cells, described by DAVIES and SMALL [13] in the neutrophil leucocytes of the lamprey as "envelope-limited sheets of chromatin". Many of such chromatin sheets were encountered in the fish leucocytes of type 1 and a few in amphibian neutrophil leucocytes, but none in the type 2 cells.

A characteristic feature of the eosinophil granules of higher vertebrates is their high isoelectric point, possibly related to an arginine-rich basic protein, which could not be separated from the peroxidase enzyme of the granules [5]. This basic protein of the rat was found to promote the phagocytic activity of monocytes, a similar protein extracted from the rabbit heterophil granules produced emigration of the leucocytes, inflammatory changes in the microcirculation of the mesenteric vessels, disruption of the mast cells and adhesions [25, 26].

The peroxidase of the neutrophils (rat, guinea-pig) reoxidizes $NADPH_2$ to NADP during phagocytosis and regulates the formation of hydrogen peroxide, necessary for the killing of ingested bacteria or for the oxidation of other ingested foreign material [23, 34]. The peroxidase of the eosinophils, the functions of which are not known, was found to be different from the neutrophil peroxidase [3]. In birds and reptiles the benzidine peroxidase reaction of

Fig. 32. Type 1 cell, tench kidney (formol–phosphotungstic acid–Araldite). Homogeneously electron-dense granules, crystalloids unstained. Arrow points to "envelope-limited sheet of chromatin". Appr. ×5,000

Fig. 33. Type 2 cell, tench kidney (OsO_4–Maraglas, uranyl acetate–lead citrate). Relatively large granules, some of them with fibrillar-granular matrix structure and electron-dense core. Appr. ×5,000

the heterophils, which are as strongly acidophilic as the eosinophil granules, is negative; in fish leucocytes the granules with a positive benzidineperoxidase reaction do not show a high isoelectric point. These observations seem to suggest that the strongly basic protein responsible for the high isoelectric point of the heterophil and eosinophil granules and the benzidineperoxidase enzyme are different substances. Previously it was suggested [30] that the high isoelectric point of the eosinophil granules might be due to their peroxidase protein, since myeloperoxidase, according to AGNER [1], has a strikingly high isoelectric point, at pH 10.5. In the light of our present observations this assumption seems unlikely.

In spite of the fact that the alkaline erythrosin and anisotropic Congo red or Sirius red F3B as well as the fluorescent thioflavine S stainings showed the same selectivity, their underlying mechanism of binding differ [28, 29]. The positivity of alkaline erythrosin staining is indicative of a basic protein of high isoelectric point, i.e., of basic groups able to bind acid dyes by strong salt-like, or ionic bonds even at an alkaline pH. On the other hand, Congo red, Sirius red F3B and thioflavine S are substantive dyes, whose elongated and flat (coplanar) molecules are bound to the tissue structures, like to cellulose microfibrils, by a large number of non-ionic linkages. The condition for such a combination of dye and structure is the oriented pattern of the structure with a sufficiently high number of reactive groups. Thus, alkaline erythrosin staining is indicative of a chemical rather than structural, whereas Congo red, Sirius red F3B and thioflavine S staining of a structural rather than chemical feature. The close parallelity of the two staining reactions suggests that the same substance may be responsible for both, i.e., the strongly basic protein of the granules may be of a submicroscopic oriented structure.

Our electron microscopic observations have revealed a number of common features of the leucocytic granules of lower vertebrates. They are invariably electron-dense structures, surrounded by a single membrane and stained by phosphotungstic acid; they seem to originate in the Golgi vesicles of the cells. Generally heterophil (neutrophil) granules are more variable in shape than the eosinophils.

The granule-matrix is usually homogeneous, but certain conditions (fixation, solvent effects, cellular breakdown, enhanced functional activity) will lead to a granular, vesicular, or fibrillar matrix-transformation. In nearly all types of granules such structural transformation of the matrix may occur (Table 1). These changes in the granule-matrix remind of the alteration of the matrix of human and rat eosinophil granules, described as "granular-vesicular matrix transformation" [31], which seems to be associated with an enhanced activity of the granule enzymes [35]. According to our present observations such changes are common in both the eosinophil and heterophil (neutrophil) granules. This masked structure of the granules may account

for their anisotropic staining reactions with substantive dyes. Further studies are needed, however, first, for differentiating artefacts due to fixation, staining, etc., and pathophysiological alterations, and secondly, for a better understanding of the functional morphology of the leucocytic granules.

BRO-RASMUSSEN and EGEBERG [8] described peculiar structures, indentations or excavation-like holes at the periphery of the matrix in the granules of rat eosinophil leucocytes. Such structures were frequently encountered in the amphibian eosinophil granules and regularly observed in the heterophil granules of reptiles. Although their function is not known, the electron microscopic observations suggest that they may represent sites of interaction between the granules and the cytoplasm.

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VERGLEICHENDE HISTOCHEMISCHE UND ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNG DER EOSINOPHILEN LEUKOCYTEN IN WIRBELTIEREN

I. Untersuchungen an Vogel-, Reptilien-, Amphibien- und Fisch-Leukocyten

Die Leukocyten im Blut und in den hämopoetischen Geweben von niederen Wirbeltieren (Vögeln, Reptilien, Amphibien, Fischen) wurden im Licht- und Elektronenmikroskop untersucht. Die Granula von Vögel-, Reptilien- und Amphibien-Leukocyten zeigten einen hohen isoelektrischen Punkt (alkalisches Erythrosin), anisotrope Färbung mit Kongorot oder Siriusrot F3B, Fluoreszenz mit Thioflavin S und positive neutrale und alkalische Benzidinperoxidasen-Reaktion. Bei Fischen (Karpfen, Karausche und Schleie) wurden zwei Leukocytentypen festgestellt. Keine ihrer Granula wies einen hohen isoelektrischen Punkt auf, oder zeigte eine Kongorot- bzw. Siriusrot-F3B-Anisotropie, noch eine Thioflavin-S-Fluoreszenz. In der einen leukocytären Zell-Linie gaben indessen die Körnchen eine positive neutrale Benzidinperoxidase-Reaktion (Zellentyp 2). In der anderen Zell-Linie werden sowohl die neutrale als auch die alkalische Reaktion negativ (Zellentyp 1). Die letzteren Zellen enthielten zahlreiche kleine Granula und wurden als neutrophile Leukocyten klassifiziert. In den Zellen des Typs 2 war die Anzahl der Benzidinperoxidase-positiven Granula geringer und ihre Maße waren relativ größer. Es wird angenommen, daß sie »eosinophile« Leukocyten sind. In sämtlichen untersuchten Wirbeltierarten zeigte die Matrix der leukocytären Granula eine granuläre oder fibrilläre Umwandlung, die an die ähnliche Veränderung der eosinophilen Granula in Ratten oder am Menschen erinnerten. Die Umwandlung der Matrix ist vermutlich ein Zeichen erhöhter funktionaler (enzymatischer) Aktivität. In den Leukocytenkörnchen von Amphibien und Reptilien waren häufig Indentationen bzw. exkavationsartige Vertiefungen zu sehen, die vermutlich den Ort der Wechselwirkung zwischen den Granula und dem Cytoplasma andeuten.

СРАВНИТЕЛЬНОЕ ГИСТОХИМИЧЕСКОЕ И ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ ЕОЗИНОФИЛЬНЫХ ЛЕЙКОЦИТОВ У ПОЗВОНОЧНЫХ

I. Изучение лейкоцитов птиц, пресмыкающихся, земноводных и рыб

Лейкоцитов крови и кроветворных тканей низших позвоночных (птиц, пресмыкающихся, земноводных и рыб) были изучены в световом и электронном микроскопе. Зернышки эозинофильных лейкоцитов птиц, пресмыкающихся и земноводных показывали высокую изоэлектрическую точку (щелочный эритрозин), анизотропное окрашивание кон-

гокрасным или сириус красным F3B, флюоресценцию с тимофлавином S и положительную нейтральную и щелочнобензидин-пероксидазную реакцию. У рыб (карпы, речные уклейки и лини) были выявлены два типа лейкоцитов. Ни одно из зернышек не показало высокой изоэлектрической точки, анизотропии с конгокрасным или сириус-красным F3B, или флюоресценции с тиофлавином S. Однако в одной лейкоцитарной клеточной линии зернышки давали положительную нейтральную бензидин-пероксидазную реакцию (клеточный тип 2). В другой линии были получены отрицательные нейтральные и щелочные реакции (клеточный тип 1). Последние клетки содержали значительное число мелких зернышек и в них часто встречались «ограниченные оболочкой хроматиновые пластинки». Эти клетки классифицировали как нейтральные лейкоциты. В клетках типа 2 число зернышек, дающих положительную бензидин-пероксидазную реакцию, было меньшим, и их размеры оказались относительно большими. Полагается, что эти клетки представляют собой «эозинофильные» лейкоциты. Во всех исследовавшихся видах животных матрикс лейкоцитарных зернышек показал зернистое или фибриллярное преобразование, напоминающее подобное изменение эозиномильных зернышек у крысы и человека. Преобразование матрикса авторы рассматривают как знак усиленной функциональной (энзиматической) активности. В лейкоцитарных зернышках пресмыкающихся и земноводных часто наблюдались индентация или углубления в виде экскавации, повыдимому указывающие на место взаимодействия между зернышками и цитоплазмой.

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FLUORESCENCE HISTOCHEMICAL STUDIES ON THE PREOPTIC RECESS ORGAN IN VARIOUS VERTEBRATES

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Synopsis

Brains of fishes, amphibians, reptilia, birds and mammals were studied with routine histological methods and by fluorescence histochemistry for the demonstration of monoamines. In the rostral part of the preoptic recess of urodela and anura (*Amblystoma mexicanum*, *Pleurodeles waltlii*, *Rana esculenta*, *Rana ridibunda*, *Bufo bufo*, *Bombinator igneus*), a special area, the so-called preoptic recess organ (PRO) is composed of special ependymal cells and monoamine-containing neurons. The ventricular dendrites of these neurons form club-like, ciliated nerve terminals floating freely in the cerebrospinal fluid (CSF). Monoaminergic fibres seem to build up connections between the PRO and the telencephalon, the different parts of the diencephalon and the mesencephalon. Ontogenetically, the PRO appears later than the monoamine-containing paraventricular organ and the mesencephalic reticular nuclei. In the primordium of the PRO, neurons showing induced fluorescence were first observed in 27 mm long *Bufo bufo* larvae. The induced green fluorescence of the PRO increases after treatment with Nuredal, decreases after administration of reserpine, and disappears after DL-m-tyrosine injection, suggesting the presence of noradrenaline. In frogs kept in 1 per cent NaCl solution for one day, the induced fluorescence of the PRO is increased. There is no change in frogs dehydrated for six days. On the basis of the morphological characteristics, the PRO is thought to belong to the liquor-contacting neuron system, supposed to form a direct informatory link between the CSF and the neuroendocrine system.

Introduction

The recessus praeopticus organ described by us in amphibia [10, 16] is pruated in the rostral part of the preoptic recess anterior to the neurosecretory meoptic nucleus. The preoptic recess organ (PRO) is built up of special ependyttal cells and monoamine-containing neurons. The ventricular processes of ihese nerve cells protrude into the lumen of the third ventricle, where they form club-like terminals. Electron microscopic studies have revealed that the PRO resembles the structure of the paraventricular organ [8, 13, 17]. The enzyme-histochemical pattern of the organ was studied by TEICHMANN and VIGH [10] and VIGH-TEICHMANN [16]. In our previous paper [16], we reported some data concerning the phylogeny of the PRO. In the present work the morphology of the PRO of various vertebrates has been investigated with fluorescence histochemical method. The paper includes, in addition, some ontogenetic and experimental data.

Material and method

The brains of 102 animals were studied by the fluorescence microscopic method of FALCK and OWMAN [3] for the histochemical demonstration of biogenic monoamines. The material was frozen in isopentane cooled with liquid nitrogen, and then dried in a Speedivac-Pearse freeze-drying apparatus. After treatment with paraformaldehyde gas for 1 hour at 80 °C and embedding in paraffin, sections of 9 to 10 μ thickness were prepared and studied under the fluorescence microscope. The deparaffinized sections were counterstained with haematoxylin eosin. For control purposes (autofluorescence), material embedded in paraffin without previous paraformaldehyde gas treatment was studied with fluorescence microscope. Serial sections (5 μ thick) of about 60 Bouin-fixed brains *(Rana esculenta, Amblystoma mexicanum)* were stained with chrome alum gallocyanine according to BOCK [1] and with Gabe's aldehyde fuchsin [4]. Semithin sections of the PRO of amphibians *(Bufo bufo, Rana esculenta, Bombinator igneus, Pleurodeles waltlii)* fixed in Karnovsky's fixative [6] or in 2.5, 4 and 5 per cent glutaraldehyde in Millonig buffer and embedded in araldite, were stained with toluidine blue azur II.

The following species were examined:

Fish: Lebistes reticulatus, Carassius auratus, Cyprinus carpio.
Amphibia: Amblystoma mexicanum, Pleurodeles waltlii, Rana esculenta, Rana ridibunda, Bombinator igneus, Bufo bufo.
Reptilia: Natrix natrix, Emys orbicularis, Lacerta viridis.
Birds : Gallus domesticus, Passer domesticus.
Mammals: Mus musculus, Epimys norvegicus, Cavia cobaya, Erinaceus roumanicus.

Furthermore, 46 specimens of different developmental stages of *Lebistes reticulatus* (6 to 7 mm long unborn fish, 2 days old, 2 weeks old, young fish), of *Pleurodeles waltlii* (10, 11, 12 and 13 mm long, 3 weeks old) and of *Bufo bufo* (2 days old after spawning, 10, 11, 12, 13, 14, 15, 20, 21, 22, 27 mm long) were examined with the method of FALCK and OWMAN [3].

For pharmacological studies in fish, amphibians and mammals, the monoamine oxidase inhibitor (MAOI) Nuredal (500 mg/kg) was injected intraperitoneally 5 to 6 hours before decapitation. In *Rana esculenta* depletion experiments were carried out by injecting 15 mg/kg of reserpine (Rausedyl) intraperitoneally. In *Bombinator igneus*, DL-m-tyrosine (400 mg/kg) was injected intraperitoneally three times at 2-hour intervals, 6 hours before decapitation.

Moreover, we examined 13 frogs *(Rana esculenta)* fluorescence histochemically after altering their water household. The frogs were kept in 1 per cent sodium chloride solution in the dark at about 6 to 8 °C for one, three or six days, and then decapitated. Three frogs similarly dehydrated for 2 or 6 days were treated with 500 mg/kg Nuredal, 5 hours before decapitation.

Results

General morphology of the preoptic recess organ. The PRO (Fig. 1) is localized at both sides of the preoptic recess rostral to the neurosecretory, magnocellular preoptic nucleus. The PRO consists of a special, ciliated columnar ependyma, one- to two-layered, and of a parvocellular neuronal part. The latter is composed of a hypendymal cell group and a distal one, both irregularly separated from one another by a small fibrous zone, and both negative with neurosecretory staining methods. The small, bipolar neurons of the hypendymal neuron layer are to be found intraependymally and hypendymally immediately beneath the stratified ependyma (Fig. 2). The neurons of the distal part are multipolar and arranged diffusely farther away from the ventricle. Ventricular processes of both neuron groups protrude into the lumen of the preoptic recess, where they terminate as club-like nerve endings (Fig. 2) floating freely in the cerebrospinal fluid (CSF). In routine stain-

ed sections we could not follow the peripheral nerve processes passing away into the brain substance.

Fluorescence-histochemical demonstration of monoamines in various vertebrates. A bright green fluorescence can be demonstrated in the neurons of the PRO, in their ventricular dendrites and terminals as well as in their peripheral processes.

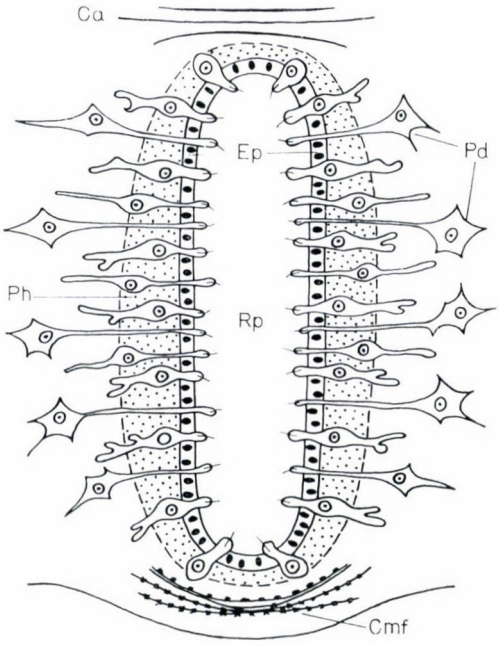

Fig. 1. Scheme of the preoptic recess organ of the frog. Ca = anterior commissure; Rp = preoptic recess; Ep = ependyma; Ph = hypendymal neuron layer; Pd = diffusely-arranged distal neurons; Cmf = crossing monoaminergic fibres

In *fish*, even after treatment with MAOI Nuredal, neither fluorescent perikarya, nor ventricular terminals could be observed in the preoptic recess area. However, a strong fibre plexus could be demonstrated around the preoptic recess and around the neurons of the neurosecretory preoptic nucleus. Furthermore, the structure and the fibre connections of the paraventricular organ were visible by brilliant yellow-green fluorescence.

In *amphibians*, the PRO was detectable fluorescence histochemically in all specimens studied. In the urodela *Amblystoma mexicanum*, the PRO (Fig. 3) contains small, fluorescent neurons lying partly close to the ependyma and partly farther away from the ventricle. The nerve cells of the hypendymal layer are bipolar. Their perikarya, ventricular dendrites, ventricular club-like terminals and peripheral processes (Fig. 3) were filled with green fluorescent material of variable amount. We could find fluorescent neurons also on the

posterior and upper surface of the anterior commissure at the foramen Monroi. Contrary to the data found in *Rana esculenta*, fluorescent nerve cells were demonstrable also around the bottom of the most rostral end of the preoptic recess. The distal neurons of the PRO were fluorescence histochemically well demonstrable especially in Nuredal-treated animals (Fig. 4). Fluorescent fibre bundles originating from the nerve cells of the PRO run in ventro-lateral

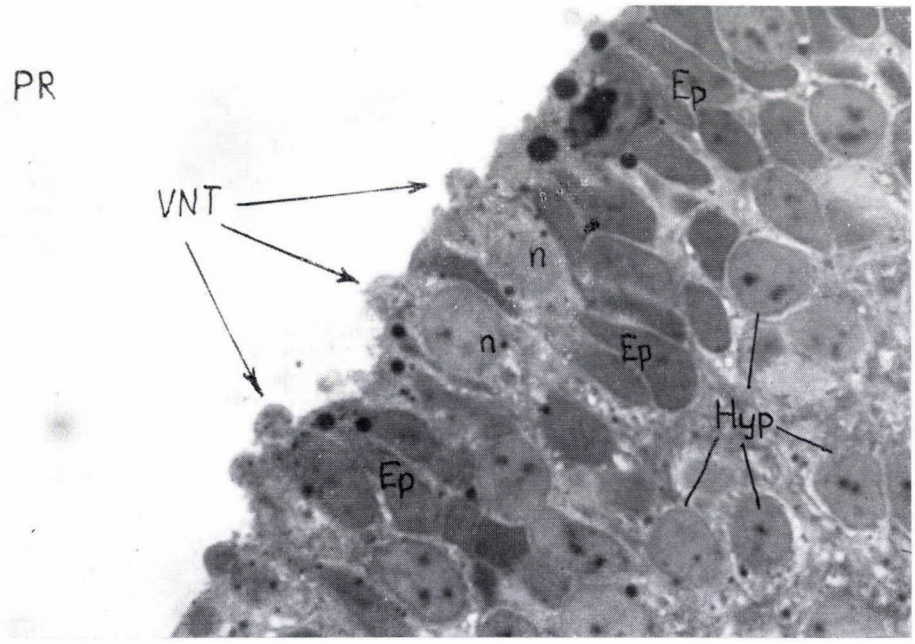

Fig. 2. Semithin section of the PRO of *Rana esculenta*. Toluidine blue azur II. Ep = ependymal cells; Hyp = hypendymal neurons; VNT = ventricular terminals; PR = preoptic recess; n = intraependymal neurons. ×1,200

direction, towards, and then beneath, the surface of the brain. They can be traced to the basal part of the telencephalon (septum, striatum). A part of the fibres are crossing ventrally from the preoptic recess and seem to pass in caudal direction above the optic tract. There were no fluorescent fibres around the neurons of the preoptic nucleus.

In *Pleurodeles waltlii*, the structure of the PRO corresponds to that of the axolotl, but there are fewer fluorescent neurons in the hypendymal and the distal cell layers, and the fibre bundles originating from the PRO are rather fine and difficult to trace. In the axolotl as well as in *Pleurodeles waltlii*, yellow fluorescent neurons (Fig. 5) belonging to the reticular nuclei, could be found in the mesencephalon. The fluorescence intensity of these nerve cells decreases quickly in UV-light, suggesting the presence of 5-hydroxytryptamine.

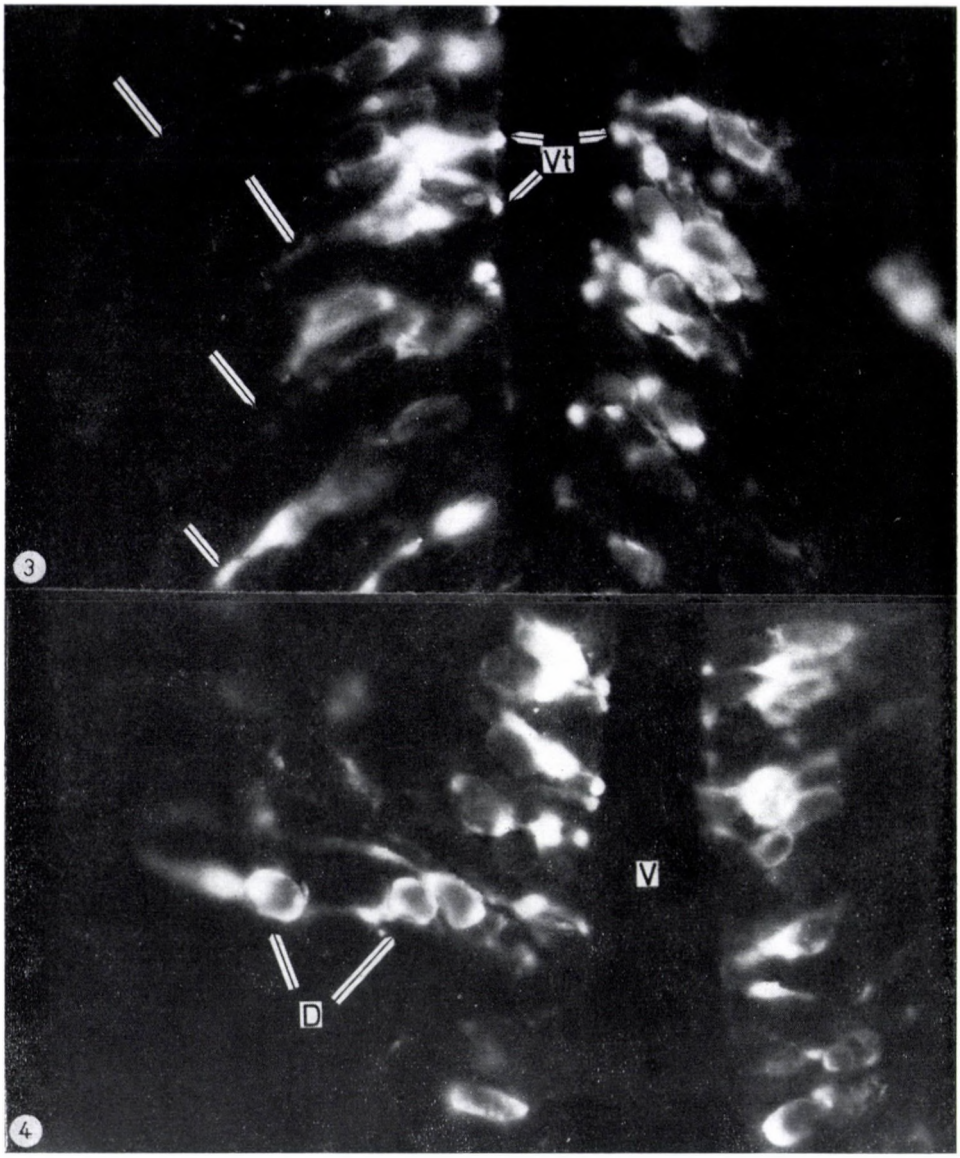

Fig. 3. PRO of the axolotl treated with Nuredal. Numerous club-like ventricular terminals (V t) around the lumen of the preoptic recess. Note the peripheral processes of hypendymal neurons. Fluorescence histochemical demonstration of monoamines. ×937

Fig. 4. PRO of the axolotl treated with Nuredal. Besides the hypendymal fluorescent nerve cells some distal neurons (D) are visible which contain monoamines. V = ventricle. ×937

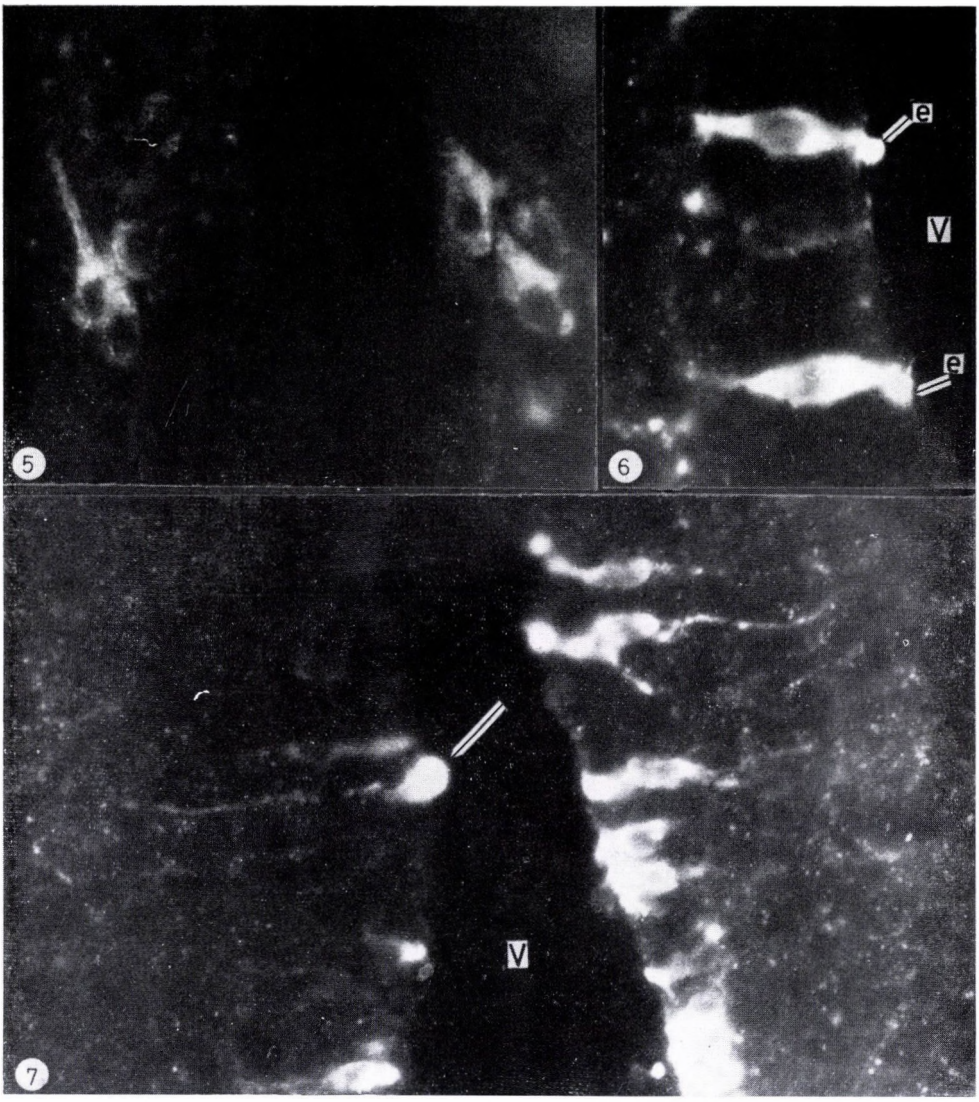

Fig. 5. Pleurodeles waltlii treated with Nuredal. The mesencephalic nucleus reticularis giving yellow induced fluorescence. ×937

Fig. 6. Bipolar hypendymal neurons forming club-like ventricular endings (e) in the PRO of the frog *(Rana esculenta)*. V = ventricle. ×937

Fig. 7. PRO of *Rana esculenta* after dehydration. The arrow points to a bipolar fluorescent cell the perikaryon of which protrudes into the ventricular lumen. V = ventricle. ×375

In all *anura* studied by us, the structure of the PRO is well demonstrable by induced fluorescence. The neurons form two cell layers, a hypendymal and a distal one. The club-like terminals of the bipolar neurons (Figs 2, 6) of the hypendymal layer are visible scattered over the whole surface of the preoptic recess, from its most rostral part up to the posterior and upper surface of the anterior commissure. The nerve cells disappear at the rostral beginning of

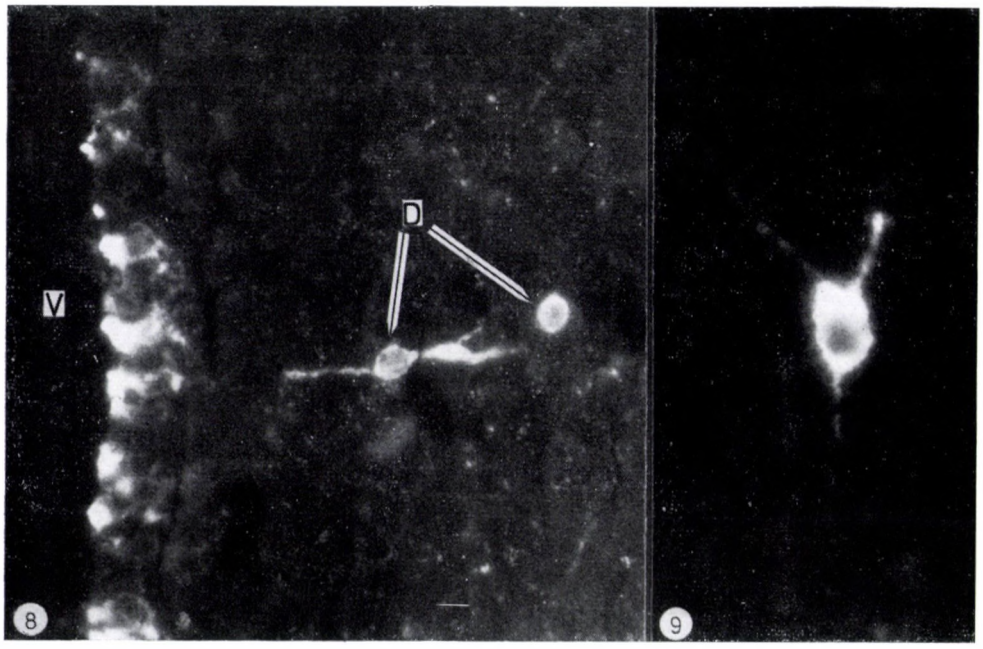

Fig. 8. PRO of a dehydrated *Rana esculenta*. Note distal fluorescent neurons (D) arranged diffusely beneath the hypendymal cell layer. V = ventricle. ×375
Fig. 9. A multipolar cell from the distal neuron group of the PRO. *Rana esculenta*. Induced fluorescence. ×937

the neurosecretory preoptic nucleus. Sometimes, the whole perikaryon of an intraependymal neuron seems to protrude into the ventricular lumen (Fig. 7). Generally, the fluorescence of the ventricular dendrites of normal frogs was more intensive than that of the peripheral cell processes. The peripheral processes of the hypendymal neurons could be traced to the distal cell layer. The nerve cells of the distal layer are arranged diffusely (Fig. 8), most of them are multipolar (Fig. 9). In semithin sections as well as in our fluorescent material, we observed distal neurons sending one of their dendrites into the lumen of the preoptic recess, where the dendrites form club-like nerve terminals (Fig. 10). Fibre bundles that originate mainly from the peripheral processes of distal neurons of the PRO cross partly below the preoptic recess (Fig. 11)

in the so-called basal part of the terminal lamina, and partly above the recess in the anterior commissure. In frontal and horizontal sections, the fluorescent fibre bundles can be traced to the telencephalon (striatum and septum), and to the diencephalon. Contrary to our findings in fish, reptilia, birds and mammals where an extended and strongly fluorescent fibre plexus surrounds

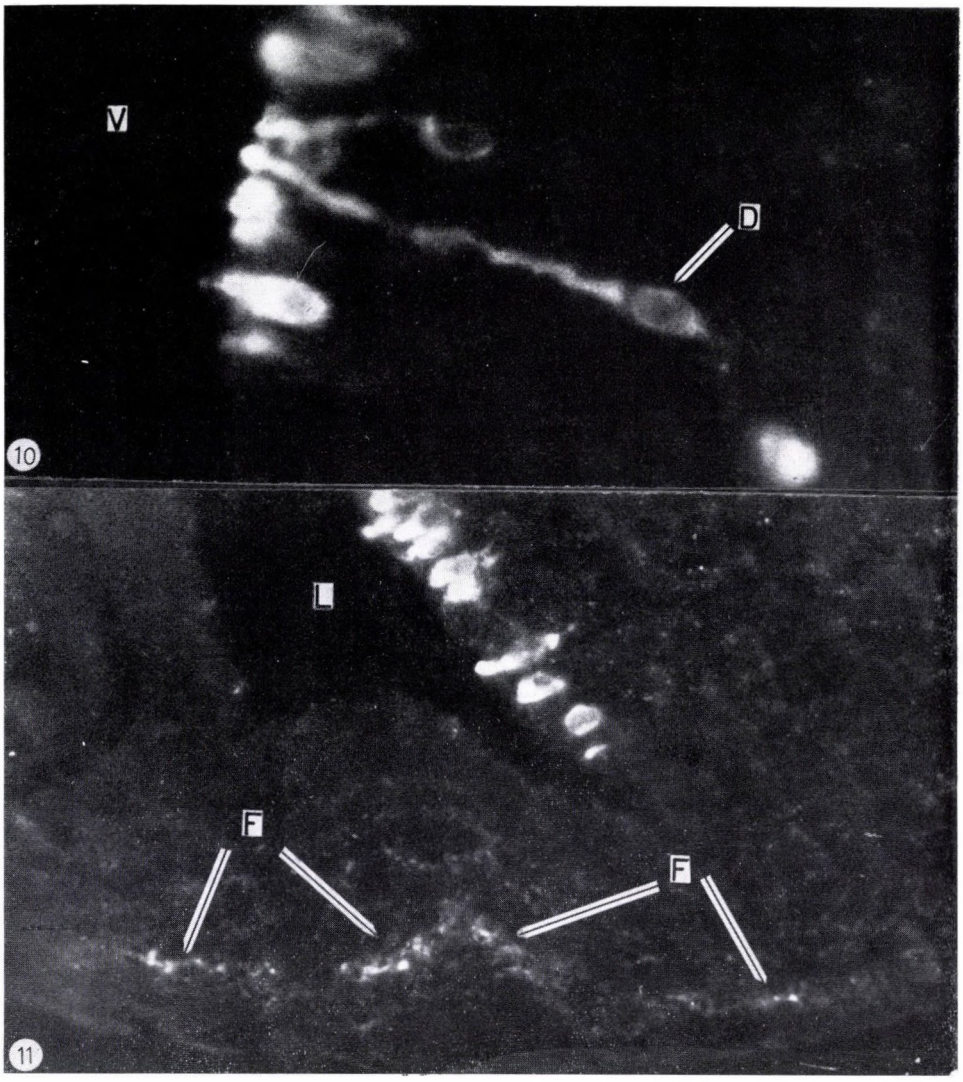

Fig. 10. PRO of a dehydrated *Rana esculenta.* Distal cell (D). Its ventricular process forms a club-like ventricular terminal in the preoptic recess. V = ventricle. ×937

Fig. 11. A part of the PRO's fluorescent fibres (F) crossing in the basal part of the terminal lamina. *Rana esculenta* treated with sodium chloride for one day. L = lumen of the third ventricle. ×375

the neurons of the neurosecretory hypothalamic nuclei (nucleus praeopticus, nc. paraventricularis and supraopticus), in amphibia only some fine fibres seem to be running to the magnocellular preoptic nucleus, and some to the epithalamus. The fibres of the PRO seem to intermingle with ascending bundles from the mesencephalic monoaminergic nucleus and from the paraventricular organ (Fig. 12).

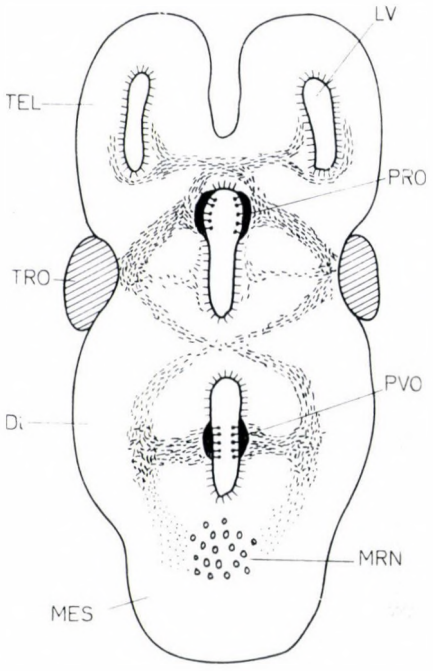

Fig. 12. Scheme of fluorescent fibre bundles of *Rana esculenta* in a horizontal section; TEL = telencephalon; Di = diencephalon; MES = mesencephalon; TRO = optic tract; LV = lateral ventricle; PRO = preoptic recess organ; PVO = paraventricular organ; MRN = midbrain-reticular neurons

In *reptilia, birds and mammals*, we could not demonstrate a PRO by induced fluorescence. In the species studied by us, the rostral part of the preoptic recess did not show any fluorescent ventricular nerve terminals or fluorescent neurons even after treatment with Nuredal. More caudally in the preoptic recess, a strongly fluorescent fibre plexus was visible periventricularly extending to the paraventricular and supraoptic nuclei. The paraventricular organ of reptilia and birds, and the mesencephalic reticular nuclei of the higher vertebrates studied by us could be well demonstrated by induced fluorescence.

Embryological development of the PRO. We failed to demonstrate any fluorescent structure comparable with the PRO of amphibia in any stages of

the fish *Lebistes reticulatus*, while the paraventricular organ shows fluorescence already in 6 to 7 mm long unborn fish. In larvae of *Pleurodeles waltlii* 10 to 13 mm long and 3 weeks old, the area of the preoptic recess is not yet fully developed and fluorescence is still lacking. The ventricular terminals of the paraventricular organ, however, show a weak induced fluorescence already in 10 mm long larvae. There were some small, yellowish fluorescent nerve cells in the mesencephalon of 11 mm long *Pleurodeles waltlii*. In *Bufo bufo*, the first bipolar neurons of the PRO could be found by their green fluorescence in 27 mm long larvae, while the induced fluorescence of the ventricular terminals of the paraventricular organ appears in 14 mm long specimens. The monoamine-containing mesencephalic neurons are detectable in 14 to 15 mm long larvae.

As regards our *pharmacological studies*, we are referring only to our observations in amphibia. Intraperitoneal injection of MAOI Nuredal resulted in a somewhat stronger green fluorescence of the neurons and nerve processes of the PRO. Especially the peripheral fibre bundles were more brilliant fluorescent in Nuredal-treated animals than in the controls. After one injection of reserpine, the intensity of the green fluorescence in the hypendymal neurons and their ventricular dendrites and terminals decreased, the fluorescence of peripheral fibre bundles disappeared almost completely. After repeated treatment with DL-m-tyrosine, a complete depletion of the monoamines could be observed in all structures of the PRO while — for comparison — the fluorescence of the paraventricular organ known as being composed of two components, a green and a yellow, only decreased. In the sections studied fluorescence microscopically without previous paraformaldehyde treatment, we failed to demonstrate the neurons of the PRO and/or their cell processes. However, it is rather interesting that the neurons of the neurosecretory preoptic nucleus and the apical cytoplasm of the ependyma of the third ventricle contained a great number of yellow autofluorescent granules. The localization of the autofluorescent particles of the ependymal cells corresponds to that of the aldehyde fuchsin positive granules studied by us histochemically and discussed as to be lysosomes [9].

The *alteration of the water household* of frogs *(Rana esculenta)* revealed the following results. After keeping the animals in 1 per cent NaCl solution for 1 day, the perikarya, ventricular dendrites and terminals of the hypendymal and distal neurons of the PRO (Figs 7, 8, 10) showed a more intense fluorescence than the same formation in untreated aminals. Furthermore, the intensity of the fluorescence of the fibre bundles originating from the PRO increased (Fig. 11) resembling the picture obtained after Nuredal injection. After treatment with NaCl for a longer period (6 days) we observed a fluorescence intensity similar to that of untreated controls. Dehydration for 2 and 6 days and additional injection of Nuredal resulted in somewhat stronger fluorescence

of the peripheral fibre bundles, compared to that of the frogs only dehydrated. It is of interest to note that the animals kept in sodium chloride solution in the dark showed a remarkable agility. Their skin became light green-yellowish-brown while that of control frogs kept in the dark was deeply greenish-brown.

Discussion

The previous fluorescence histochemical studies have shown that the preoptic recess organ is a special monoamine-containing structure situated in the rostral part of the preoptic recess of amphibia [10, 16]. It is characteristic of the PRO that it is composed of a special ependyma and of two nerve cell layers. These neurons send their ventricular dendrites into the lumen of the preoptic recess, where they form club-like endings. Our electron microscopic studies [17] have similarly shown that the nerve cells of the PRO contain dense-core vesicles, and that their dendrites build up club-like terminals possessing characteristic cilia with rather long roots and accessory basal bodies.

On the basis of our comparative fluorescence histochemical findings it can be concluded that the PRO appears first in tailed amphibia. Thus, it is a phylogenetically younger structure than other monoaminergic areas, e.g. the paraventricular organ and the reticular nuclei. This is in agreement with our embryological data: the PRO appears in a later ontogenetic stage than the paraventricular organ and the reticular nuclei [19]. In higher vertebrates (reptilia, birds and mammals), we failed to demonstrate the organ by fluorescence histochemical method. This does not mean that the whole PRO as a special liquor-contacting area is lacking, perhaps only its monoaminergic neuronal component is missing. In our recent enzyme-histochemical studies we could demonstrate this organ in reptilia (*Emys orbicularis* [20]). In this species, the PRO is characterized by hypendymal and distal neurons showing a considerable activity for acetylcholinesterase. Both cell types form ventricular nerve endings which are also acetylcholinesterase-positive. In mammals, too, ventricular nerve terminals were described in the surrounding of the foramen Monroi [7]; these endings presumably correspond phylogenetically to the upper part of the PRO of amphibians. Therefore, we think that the PRO exists not only in amphibians, but in higher vertebrates, too.

Our pharmacological studies confirm the presence of monoamines in the neurons of the PRO. As the induced fluorescence is green and can be reduced by administration of reserpine and slightly increased by Nuredal one can guess a primer catecholamine. The complete depletion of this amine after treatment with DL-m-tyrosine known as to act fairly selectively on noradrenaline [2], speaks in favour of the presence of the latter amine.

As for the function of the PRO, its similarity to the olfactory epithelium [5, 11] suggests a receptory function. However, a monoamine-secreting

activity cannot be excluded. There seems to be some connection between the PRO and the water household, too, since an acute dehydration increased the intensity of the fluorescence in the PRO. At present, we cannot decide whether dehydration acts directly on the ventricular terminals of this monoamine area *via* CSF, or whether it exerts some indirect action upon the PRO by means of nervous connections with the paraventricular organ, preoptic nucleus or epithalamus. Furthermore, fibre connections with the telencephalon and the mesencephalic monoaminergic reticular nuclei suggest a link with the ascending activating mechanism, too.

Finally, we would like to stress that on the basis of its special structure the PRO belongs to the so-called "liquor-contacting neuronal system" [10, 12, 13, 14, 15, 19, 20]. This system is composed of different neurons forming special, free, ciliated nerve endings in the CSF as e.g. the paraventricular organ and the liquor-contacting nerve cells of the preoptic nucleus and the paraventricular nucleus [10, 18, 20], etc. The function of the liquor-contacting neuronal system is probably to form a direct informatory link between the CSF and the neuroendocrine system. In further experimental studies we intend to elucidate the special function of the PRO in this system.

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FLUORESZENZHISTOCHEMISCHE UNTERSUCHUNGEN AM ORGANON RECESSUS PRAEOPTICUS BEI VERSCHIEDENEN WIRBELTIEREN

Gehirne von Fischen, Amphibien, Reptilien, Vögeln und Säugern wurden mit histologischen Routinemethoden und fluoreszenzhistochemisch zum Nachweis biogener Monoamine untersucht. Im rostralen Abschnitt des Recessus praeopticus von Urodelen und Anuren *(Amblystoma mexicanum, Pleurodeles waltlii, Rana esculenta, Rana ridibunda, Bufo bufo, Bombinator igneus)* findet man ein spezielles Gebiet, das sogenannte Recessus praeopticus-Organ (RPO), das aus speziellen Ependymzellen und monoaminhaltigen Neuronen aufgebaut ist. Die ventrikulären Dendriten dieser Nervenzellen bilden knöpfchenförmige, mit Zilien besetzte Nervenendigungen, welche frei im Liquor cerebrospinalis schweben. Monoaminerge Fasern scheinen Verbindungen zwischen dem RPO und dem Telencephalon, den verschiedenen Abschnitten des Diencephalon und dem Mesencephalon herzustellen. — Ontogenetisch gesehen erscheint das RPO später als das monoaminhaltige Paraventrikularorgan und die mesencephalen, retikulären Kerne. Nervenzellen, die induzierte Fluoreszenz aufweisen, wurden in der Anlage des RPO erstmals bei Krötenlarven *(Bufo bufo)* von 27 mm Länge beobachtet. — Die induzierte, grüne Fluoreszenz des RPO verstärkt sich nach Gabe von Nuredal, vermindert sich nach Reserpinverabreichung und verschwindet völlig nach DL-m-Tyrosininjektion; diese Daten weisen auf ein Vorkommen von Noradrenalin hin. — Bei Fröschen, die einen Tag in 1%iger NaCl-Lösung gehalten wurden, verstärkte sich die induzierte Fluoreszenz des RPO. Frösche, die 6 Tage dehydriert worden waren, wiesen keine Veränderungen im RPO auf. — Wir sind der Meinung, das das RPO auf Grund der morphologischen Merkmale zum Liquorkontakt-Neuronensystem gehört, welches vermutlich als ein Informationsglied zwischen Liquor cerebrospinalis und dem neuroendokrinen System dient.

ФЛЮОРЕСЦЕНТНО-ГИСТОХИМИЧЕСКОЕ ИЗУЧЕНИЕ ОРГАНА ПРЕДЗРИТЕЛЬНОГО УГЛУБЛЕНИЯ НА РАЗЛИЧНЫХ ВИДАХ ПОЗВОНОЧНЫХ

Головной мозг рыб, земноводных, пресмыкающихся, птиц и млекопитающих был изучен с помощью повседневных гистологических методов, а также флюоресцентной гистохимической техникой, в целях выявления моноаминов. В краниальной части предзрительного углубления urodela и anura *(Amblystoma mexicanum, Pleurodeles waltlii, Rana esculenta, Rana ridibunda, Bufo bufo, Bombinator igneus)*, особая зона, так наз. орган предзрительного углубления, образуется из специальных эпендимальных клеток и нейронов, содержащих моноамины. Желудочковые дендриты этих нейронов образуют булавовидные ресничные нервные окончания, свободно плавающие в спинномозговой жидкости. Моноаминергические волокна обеспечивают, по-видимому, связь между органом предзрительного углубления и телэнуефалоном, различными диэнуефалона и мезенуефалоном. Онтогенетически орган предзрительного углубления появляется позже чем околоже-

лудочковый орган, содержащий моноамины, и сетчатые ядра промежуточного мозга. В зачатке органа предзрительного углубления были выявлены нейроны, показывающие индуцированную флуюоресценцию в головастиках ***Bufo bufo*** длиной в 27 мм. Индуцированная зеленая флюоресценция органа предзрительного углубления после введения Нуредала усиливается, она уменьшается после дачи резерпина и снимается инъекцией DL-m-тирозина, что указывает на присутствие норадреналина. У лягушек, содержанных в течение сутки в 1%-ом растворе поваренной соли, индуцированная флюоресценция органа предзрительного углубления усиливается. При дегидратации лягушек в течение шести дней изменения флюорасценции не наблюдается. На основе морфологических признаков полагается, что орган предзрительного углубления относится к системе нейронов, соприкасающихся со спинномозговой жидкостью, по-видимому обеспечивающих непосредственную информационную связь между спинномозговой жидкостью и нейроэндокринной системой.

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ANALYSIS OF THE IN SITU ELECTRICAL ACTIVITY OF NERVES IN FRESH-WATER MUSSEL (ANODONTA CYGNEA L.)

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Synopsis

(1) The "spontaneous" electrical activity of nerves is composed of irregular unit potentials, regular rhythmic spikes originating from a small number of units, single or periodic bursts lasting several seconds and also of large, compound potentials. The amplitudes of activity are comparatively low (under 40 μV), except for "giant potentials" (40—90 μV) recorded sometimes from interganglionic connections. (2) Both afferent and efferent fibres are present in most of the peripheral nerves. The long, inhomogeneous bursts originate in the ganglia, others in peripheral structures. After cutting the posterior pallial nerve considerable changes were observed in the activity pattern recorded from the periphery. No afferent impulses were found in the nerves of the anterior adductor. (3) Transmission of burst from peripheral to interganglionic nerves is accompanied by a decrease of the amplitudes of spikes and length of the burst; a reverse phenomenon occurs during transmission from interganglionic nerves to the periphery. The ganglionic transmission lasts about 180—190 msec. (4) Both adaptation and selective block of ganglionic transmission were observed. This latter may play an important role in the coordinative functions of the ganglia, and refers to the presence of inhibitory mechanisms. Potentials were recorded extracellularly throughout.

Introduction

One of the essential functions of the CNS is the working up of peripheral information and further to direct the signs or patterns developed spontaneously or formed under the influence of afferent impulses towards adequate pathways. Synchronous recording of activity from different nerves may be effective in obtaining data to understand several points of coding and the neural organization within the CNS. Investigation of the electrical activity may furnish information also about the functional structure and fibre composition of the nerves themselves.

There are several data on the electrical activity of the peripheral nervous system in Pelecypoda. PAVLOV [10], ZHUKOV [19], BARNES [2] and SALÁNKI and co-workers [14] suppose the presence of stimulatory and inhibitory centres in the CNS and also the existence of at least two kinds of fibres in the innervation of the adductor muscles. PUMPHREY [11] and HORRIDGE [4, 5] succeeded in differentiating several groups of fibres in peripheral nerves of marine mussels. The different components of the evoked, compound potential of the cerebrovisceral connective in *Anodonta* was explained with differences in fibre diameters by LÁBOS and co-workers [7]. The effects of electrical, chemical and

light stimuli were also investigated in fresh-water and marine species [1, 6, 16, 17, 18].

In spite of the above-mentioned data, our information is very limited about the relation between the activity of different nerves under unaltered and stimulated conditions (spontaneous and evoked activity, respectively). Our aim was to investigate these relations partly in specifically not affected

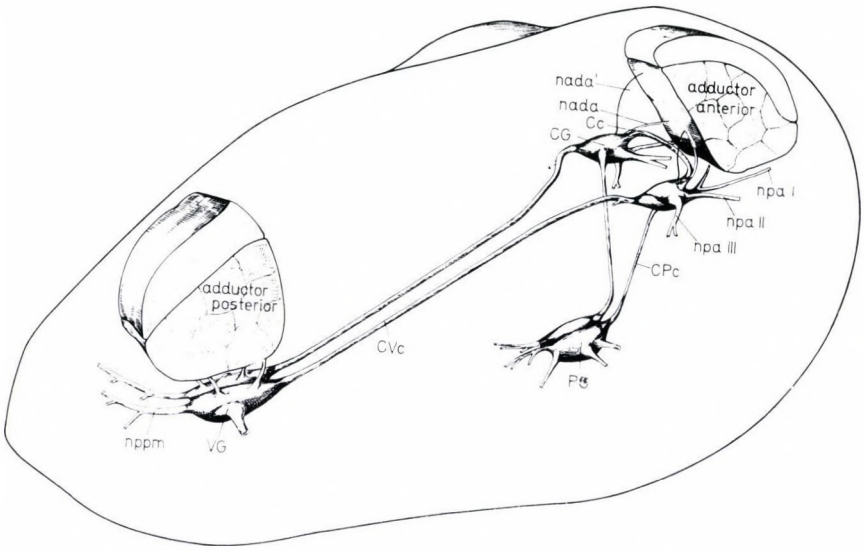

Fig. 1. Schematic arrangement of ganglia and the main nerves in *Anodonta cygnea* L. The proportions are not valid. VG = ganglion viscerale; CG = ganglion cerebrale; PG = ganglion pedale; nppm = n. pallialis posterior maior; CVc = cerebro-visceral connective; CPc = cerebro-pedal connective; Cc = commissura cerebro-cerebrale; npa = n. pallialis anterior (I, II and III); nada = n. adductoris anterioris

animals, partly in animals stimulated through mechanoreceptors. Also some problems of ganglionic transmission were examined.

Material and method

Anodonta cygnea L. specimens, 16—20 cm in length were used. The adductors were cut and one of the shells was removed. The visceral ganglion (VG) and one of the cerebral ganglia (CG) were made free from the connective tissue. Also nn. palliales posteriores maiores (nppm), nn. palliales anteriores (npa I, II, III), cerebro-visceral (CVc) and cerebro-pedal (CPc) connectives, commissura cerebro-cerebralis (Cc) and two nerves running to the adductor anterior (the thicker — nada, the thinner — nada' Fig. 1) were prepared.

Bipolar platinum electrodes 0.2—0.5 mm in diameter were used. There was one mm distance and 50—200 KΩ resistance between the two wires. In case the nerves were very thin, we used suction electrodes. Registration took place with an 8-channel electroencephalograph (Typ EMG MB 5206), which has symmetrical input with 2 MΩ impedance. In other

cases DISA EMG amplifier and Universal Indicator were used with photorecorder. The noise was usually 2—7 μV.

The preparation was protected from desiccation, using physiological saline [8], and we did not left the nerves on air longer than for 5—6 minutes. In such conditions each animal could be used throughout 7—12 hours.

Results

Characteristics of the in situ activity in different nerves

Three activity types were recorded both during spontaneous and evoked activity: (a) regular or irregular unit potentials, (b) series of single spikes (bursts) appearing sometimes in sequences, (c) compound potentials.

The amplitudes of unit potentials varied between 10 and 90 μV, the highest value being different in different nerves. The compound potentials usually reached several hundred μV.

The duration of the unit and compound potentials were 10—60 msec and 1—2 sec, respectively, — this was characteristic of different nerves. Although the values mentioned below and belonging to a given nerve are of general validity, there may occur individual deviations.

Nervus pallialis posterior maior

The spontaneous activity consists of unit potentials being 8—20 μV in amplitude, 10—15 msec in duration, 0.5—10 cps in frequency and of 4—12 sec periodic bursts characterized with 20—40 cps in frequency appearing in 1—3 min intervals (Fig. 2a). There are usually separated unit potentials of different amplitudes within a burst, however, sometimes superposition of spikes occurs. For this very reason the burst's amplitude is higher than that of the unit potentials. Train activity of 5—30 cps lasting 0.5—3 sec and repeating with 3—30 sec intervals were also observed. In this case the unit potentials originate from one or a few cells (Fig. 2b, c, d). Compound potentials are not characteristic in rest, but they could be evoked by the mechanical stimulation of the mantle margin. Its amplitude reached several hundred μV, depending on the intensity of the stimulation. No simultaneous changes were observable in the amplitudes of the unit potentials.

Nervus pallialis anterior I, II, III

Spontaneous irregular unit potentials of 8—30 μV amplitude, 10—15 msec duration and 5—15 cps frequency were recorded. Also periodic bursts

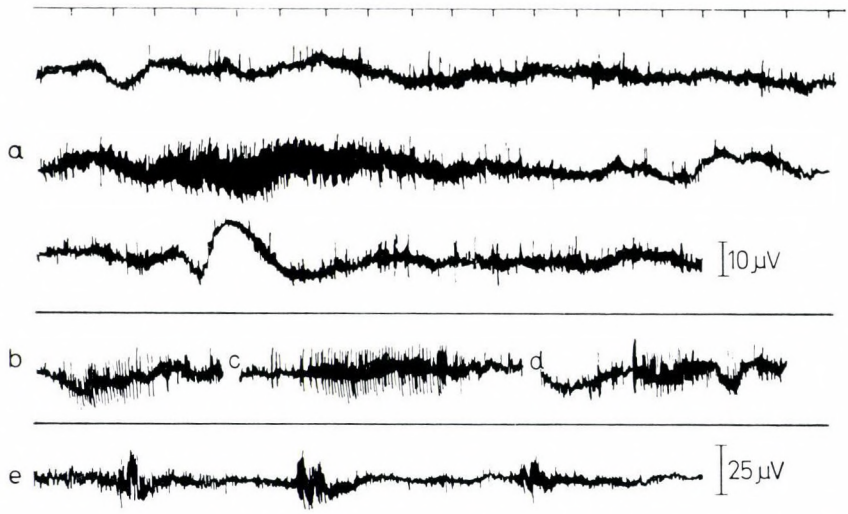

Fig. 2. a: spontaneous activity from the nppm, with burst; b, c, d: trains of unit potentials from the nppm; e: spontaneous activity from the npa I, with periodic bursts; time scale: 1 sec

of 1—3 sec duration with 10—60 sec sequences were observed from each of these nerves (Fig. 2e). Bursts were sometimes followed by a fall of the spontaneous activity (Fig. 6d).

Nervus adductoris anterioris

The amplitude of the spontaneous spikes is 10—40 μV, the frequency varies between 5 and 25 cps (Fig. 5a). Also periodic bursts and compound potentials with high amplitudes are observed (Fig. 10b).

Interganglionic nerves (CVc, CPc, Cc)

Most of the unit potentials are 10—30 μV in amplitudes, however, the duration of impulses being 20—60 msec differs from that of the peripheral nerves. Moreover, from the CVc and Cc also high, so-called "giant potentials" could be recorded.

The amplitude of the "giant potentials" from the CVc reaches 40—70 μV; they may be single impulses, or groups of two or three impulses and may follow each other at irregular intervals (several sec). On the contrary, the interval between the units of the double and triple impulses is rather constant, about 130 msec (Fig. 8).

The amplitude of the "giant potentials" recorded from the Cc reaches 60—90 μV. These may also be single, double and triple impulses. The intervals between groups are several sec, between units about 22 msec.

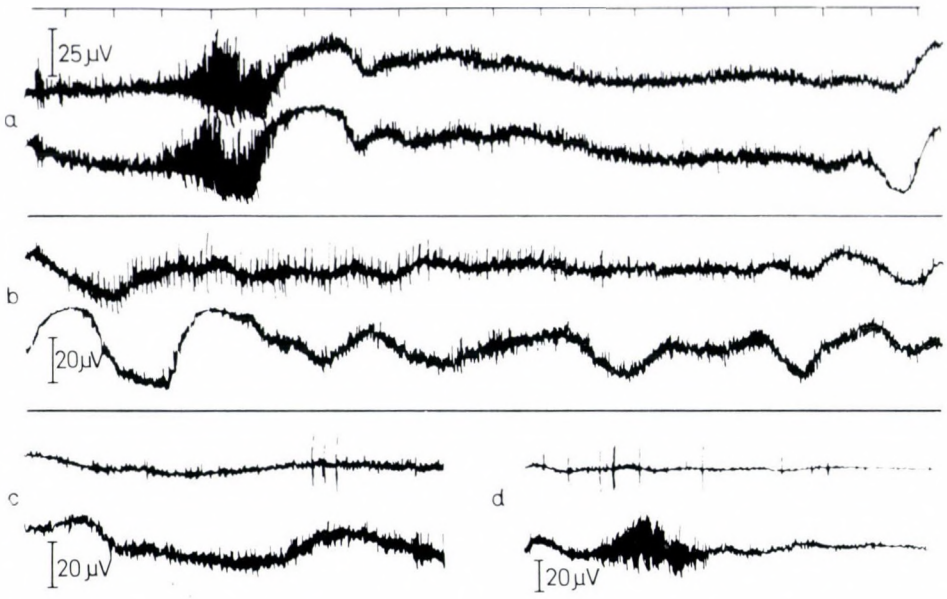

Fig. 3. Spontaneous activity of the nppm recorded with two pairs of electrodes. a: control from the intact nerve and after cutting through in the 5th second; b: after 10 sec; c: after 60 sec; d: after 120 sec; upper record: distal end, lower record: central end; time scale: 1 sec

The "giant potentials" lasted 9—12 msec in both of the nerves. Similar potentials never could be recorded from the CPc.

Origin of the activity recorded from different nerves

In situ recordings from an intact nerve give no information whether the electrical activity is of central or peripheral origin. To clear up this question, we carried out experiments with simultaneous recordings partly from paired nerves, partly from the central and the peripheral pieces of cut nerves.

It was found that the spontaneous activities of the two nppm are asynchronous in general, only the bursts lasting 4—12 sec are synchronous in the two nerves. This fact refers to the central origin of the burst activity. In contrast to this, trains composed of unit potentials of one or a few cells appear asynchronously, proving their peripheral origin.

When recording was made with two pairs of electrodes from one of the nppm, and the nerve was cut between the electrode pairs, it was found an immediate increase both in the frequency and amplitude of the unit potentials recorded from the distal part (Fig. 3b). After 15—20 sec the activity gradually decreased, remaining only unit potentials with irregular, low frequency (Fig. 3c). Burst activity was recorded only from the central end (Fig. 3d).

On the other hand, as it was expected, the stimulation of the mantle caused an increased activity only at the distal end.

There was no noticeable change in the activity recorded from the central piece of the npa I, II and III (Fig. 4b); also the periodic bursts remained intact. Unlike this, the activity of the distal end (Fig. 4 d) significantly exceeds the activity of the intact nerve. No burst activity was recorded from the distal piece in rest, but it could be evoked with mechanical stimulation of the mantle (Fig. 4e).

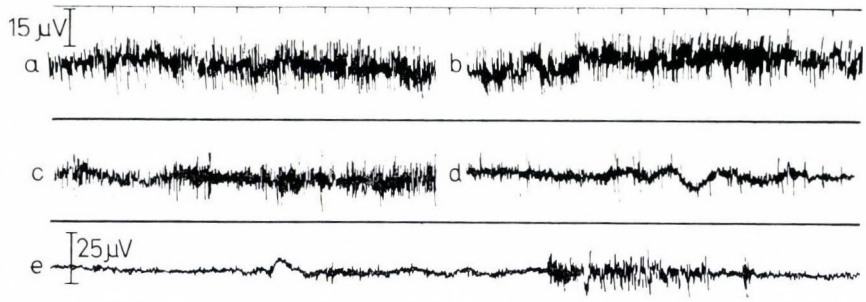

Fig. 4. Spontaneous activity from npa III. a and c: before cutting; b and d: after cutting, recorded from the central and distal end, respectively; e: burst activity from the distal end of npa I; time scale: 1 sec

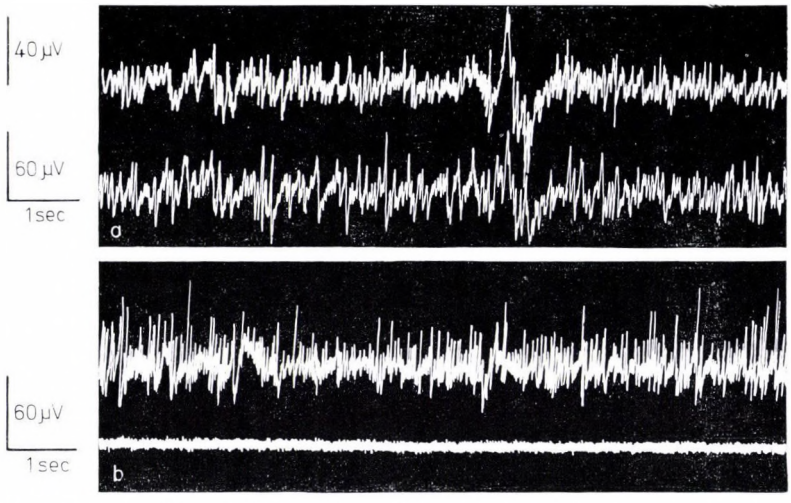

Fig. 5. Spontaneous activity of the n. adductoris anterioris. a: simultaneous registration from the central end of the nada (upper) and nada' (lower); b: after cutting the nada; upper: central end, lower: distal end

The activity of the nerves running to the adductor anterior (nada and nada') is of central origin. After cutting the nerve the activity of the central part was similar to that of the intact nerve, while no activity was recorded from the periphery (Fig. 5b).

Role of ganglia in the transmission of impulses

Besides generation of spontaneous activity, ganglia play an important role in transforming the signals arrived from the periphery or generated centrally and directing them to adequate channels. Some characteristics of the mode of operation could be cleared by simultaneous investigation of activity

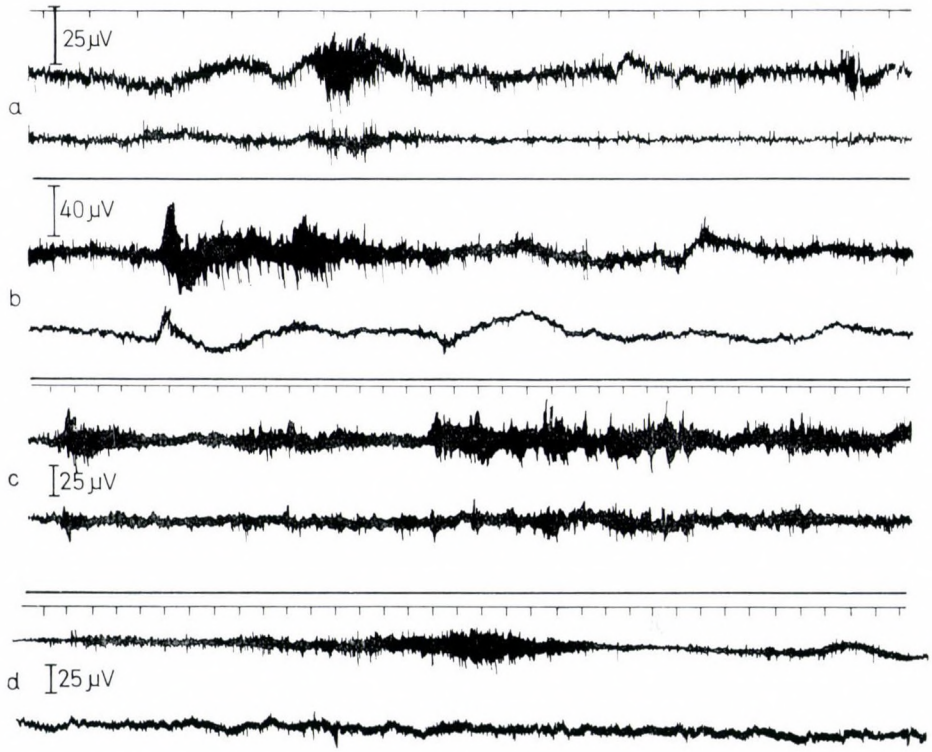

Fig. 6. Simultaneous registrations from a: npa I and CVc; b: npa II and Cc; c: npa III and Cc; d: npa III and Cc; upper records: npa; time scale: 1 sec

in different nerves. For comparison, we usually recorded the activity of the CVc parallel with the activity of one of the other nerves. Also the transmission of burst evoked by peripheral stimulation was investigated.

It was found that periodic bursts originating from the cerebral or visceral ganglia may be conducted simultaneously to several directions. When they appeared both in peripheral and interganglionic nerves, there were significant differences in intensity, the duration and amplitude being more expressed on the peripheral nerves (Fig. 6a, b, c). In other cases bursts were observed only on peripheral nerves (Fig. 6d).

Simultaneous recordings from interganglionic nerves showed that sometimes the burst could be recorded with variable intensity from all of them (Fig. 7a), while in other cases the burst could be observed but on a single nerve (Fig. 7b). Giant potentials recorded from the CVc and Cc seem to be independent from each other (Fig. 8a, b).

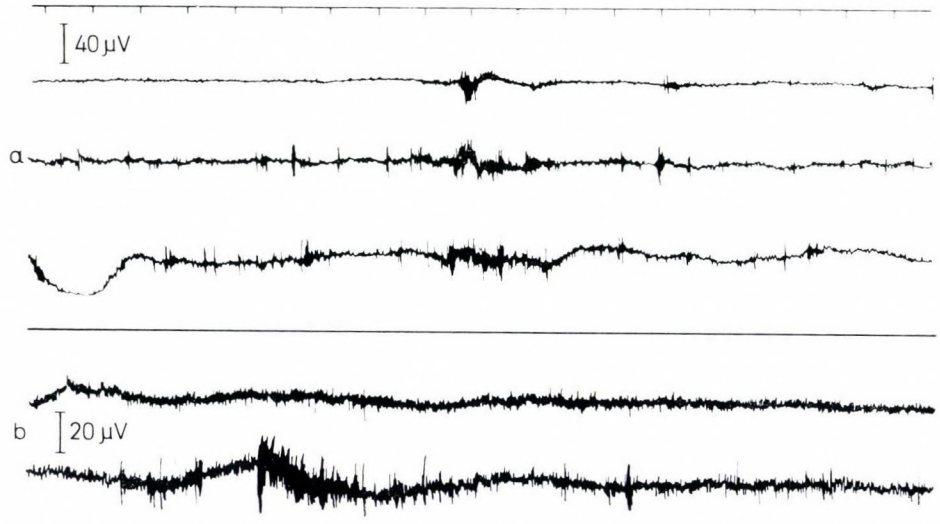

Fig. 7. Transmission of bursts originating from the CG to different nerves. a: burst is present in all the three main nerves (upper: CPc, middle: CVc, lower: Cc); b: burst appears only in the Cc (upper: CVc, lower: Cc); time scale: 1 sec

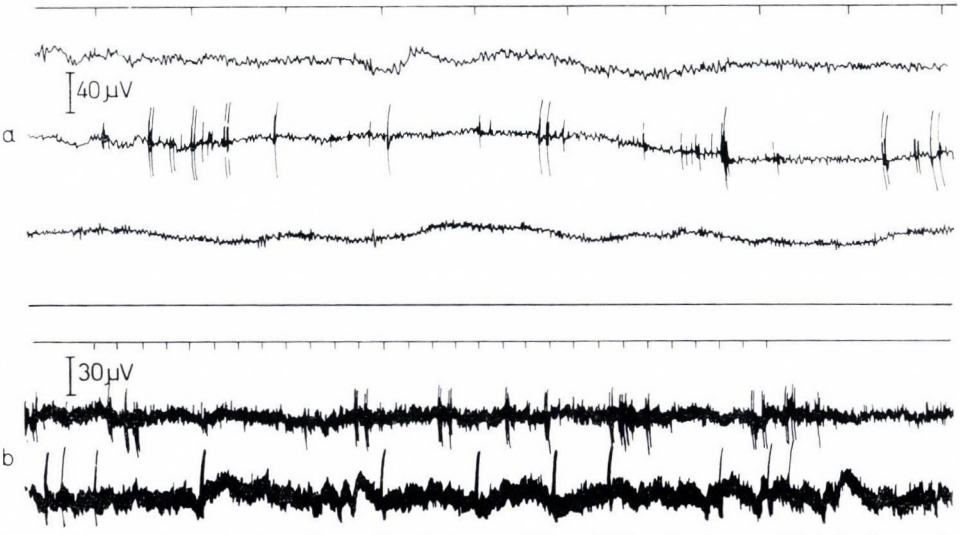

Fig. 8. "Giant potentials". a: from the Cc (upper: CVc, middle: Cc, lower: CPc); b: from the CVc (upper) and Cc (lower record); time scale: 1 sec

When burst was evoked with mechanical stimulation of the region of the sypho (posterior part of the mantle), an adequate activity was recorded in some cases in all the nerves investigated by us, whereas in other cases impulse was conducted only towards certain pathways. This shows that in the ganglia both transmission and modulation take place, i.e. the impulsation changes — increases or decreases — in intensity while being transmitted. The burst evoked by stimulation of the mantle becomes shorter while transmitted in the VG, but subsequently recorded from the npa after transmission

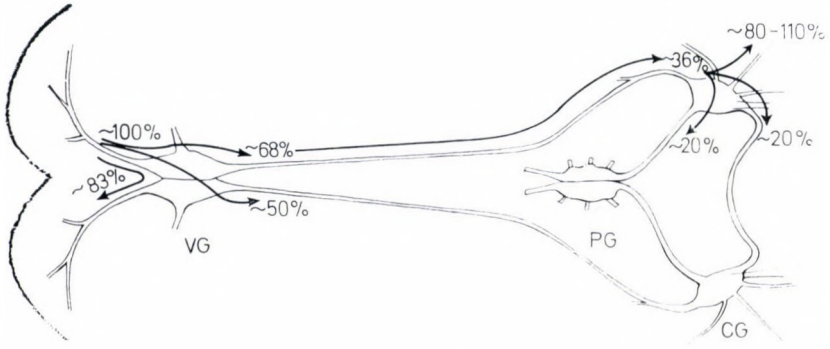

Fig. 9. Modification during transmission of the duration of the burst evoked with mechanical stimulation of the mantle. Values are given in per cent of the duration of the burst recorded in the nppm

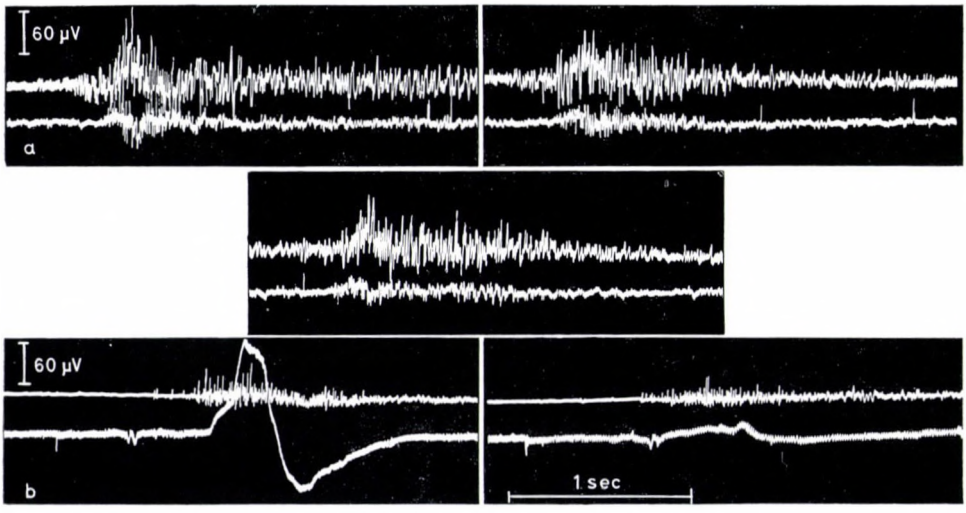

Fig. 10. Adaptation in the ganglia to repeated stimulation at 10 sec intervals. a: in the VG (upper: nppm, lower: CVc); b: in the CG (upper: CVc, lower: nada)

in the CG, it is longer again. The changes of the burst length during transmission are summarized in Fig. 9.

Passing through the VG or CG is accompanied by a delay of 180—190 msec. This is another evidence of ganglionic transmission. Obviously, to this long delay retarding processes other than synaptic delay (inhibition) must contribute.

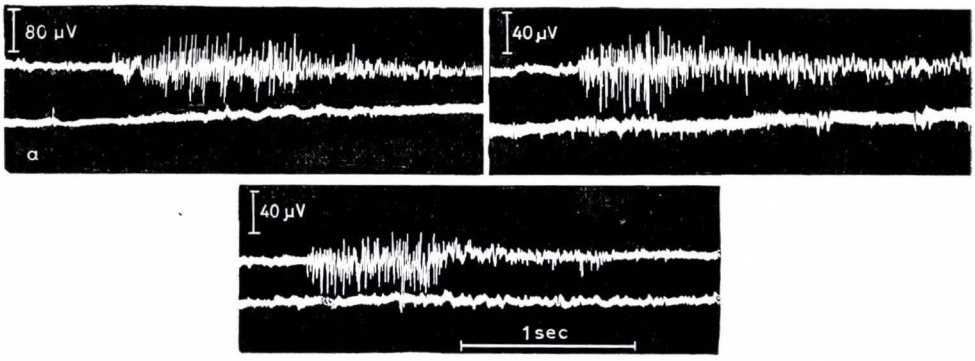

Fig. 11. Block of transmission. a: in the CG (upper: CVc, lower: Cc); b: in the CG (upper: CVc, lower: ipsilateral CPc); c: in the VG (upper nppm, lower: ipsilateral CVc)

After repeated stimulation also adaptation of neural processes was observable in the ganglia. Figs 10a and b show that, if stimulations repeatedly occur at 10-second intervals, both the compound potential and the burst will decrease. This decrease is more expressed in the postganglionic than in the preganglionic part, due to adaptation of the receptor. In other cases there is no transmission at all even at the first time, referring to the presence of inhibition in the ganglia (Fig. 11).

It was found that inhibitory processes may be selective in both the VG and CG: time and degree of inhibition may vary in different pathways. This means that inhibition is direction-sensitive (Fig. 12). In different specimens, and also in the same specimen, the observations were often different if transmission was tested at long intervals.

After mechanical stimulation the potentials from the nppm were transmitted, in most of the cases, to the ipsilateral CVc, but sometimes also to the contralateral side. In special cases inhibition was observed in both directions. Also ganglionic delay was always present, assuring the lack of direct fibres between nppm and the CVc. On the other hand, we could never demonstrate inhibition between the two npp, suggesting that there are direct fibres originating from peripheral nerve cells and running through the VG without synaptic interruption.

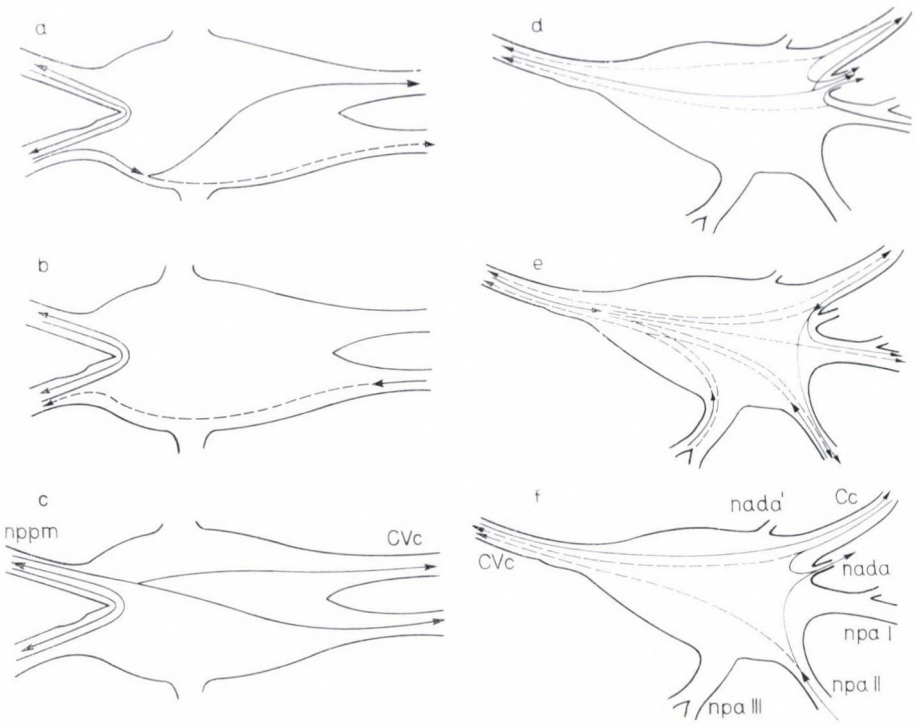

Fig. 12. The presence or lack of transmission in the VG and CG in different cases. Investigated pathways indicated. Solid line: normal transmission; broken line: inhibited transmission

Discussion

The amplitudes of unit potentials recorded with extracellular electrodes proved to be — in agreement with other data [4, 16, 17] — comparatively low. However, the frequency of the activity, the variety of amplitudes recorded from each nerve, the presence of trains and sometimes periodic bursts argue a complex, selective afferent and efferent organization.

Most of the potentials recorded from different nerves were under 40 μV; from two interganglionic nerves (CVc and Cc) also larger (40—90 μV) spikes were obtained. The size of nerve cells, being with some exception under 20—30 μ in diameter [3] and also the 0.5—1.5 μ thickness of the nerve fibres [7] furnish some explanation for the low amplitude of the spikes. MELLON [9] described also comparatively small membrane potentials (30—60 mV) in a marine Pelecypoda, suggesting a low ion-gradient between the extra- and intracellular fluid. It may be supposed that the "giant potentials" originate from larger cells, and as they never occur in peripheral nerves, may play a coordinative role between ganglia.

Unit potentials lasted in general 9—12 msec, however, they reached in the interganglionic nerves sometimes even 20—60 msec. Presumably the potentials of longer duration belong to cells taking part in central organization which, however, are not identical with the cells discharging "giant potentials". The fact that both "giant potentials" and potentials of long duration were recorded exclusively from interganglionic nerves underlines the differences between peripheral and "central" nerve paths both in fibre composition and in functional aspects.

Both the spontaneous and the evoked activity contained superposed and compound potentials of variable size. Superposition comes from joint registration of two potentials following each other in a short interval, and so this is a registration artefact. The compound potential, growing to a few mV in amplitude, may contain more than one component, and is in several respects similar to the electrically evoked potential of the isolated CVc [14]. It is the result of the synchronous discharging of several ten or hundred cells and is suggestive of intraganglionic spatial and temporal summation. According to our data the spontaneous activity of the nerves takes its origin not only in the ganglia, but, with the exception of the nerves running to the anterior adductor, it contains also centripetal impulses. Latter may originate either in receptors or in the spontaneous activity of nerve cells present at the periphery. In other molluscs nerve cells were demonstrated in peripheral structures [15], but possibly the primary receptors maintain a constant activity in the afferent nerves under our experimental conditions.

After cutting through the anterior pallial nerves, there was no change in the irregular centripetal impulsation. In contrast to this, a characteristic change was observed under similar conditions in the spontaneous activity of the distal end of the posterior pallial nerve: the frequency increased, and also potentials of higher amplitudes appeared. A few minutes later the frequency decreased, but the high amplitudes did not disappear. It may be supposed that in intact animal the ganglia suppress the functioning of certain peripheral cells or receptors, and the disconnection eliminates this inhibition. Naturally, the activation of these cells may also occur under certain physiological conditions, possibly due to some specific function of the posterior pallial area of the mantle. It is also possible that in this way the excitability of the latter structures is controlled by the VG.

In resting animals the burst originate in the ganglia. This finding is in agreement with the data of ARTEMJEV [1], who recorded bursts from isolated ganglia of *Anodonta*, and also with the results of HORRIDGE [4, 5], who evoked bursts from peripheral nerves with electrical stimulation of the CVc in *Mya*. At the same time one can elicit afferent bursts with adequate stimulation of the mechanoreceptors. The burst of peripheral origin is transmitted in the ganglia, and this is accompanied by a delay and also by changes in frequency,

duration and amplitude. Obviously, modulation of peripheral signals is significant in the ganglia, and carrying of information along interganglionic nerves requires less impulse-amount than along peripheral nerves.

Bursts of ganglionic origin being conducted — as a rule — towards several nerves, may play a role in co-ordination. On the other hand, directed transmission and conduction were sometimes observed, giving evidence of selective, discriminating capacity of the ganglia. The lack of transmission to one or other direction may be interpreted in some cases as adaptation, in others as inhibition. All these results show that CNS of *Anodonta* is not a simple reflex-centre, but it is capable of manifold, well-differentiated, regulatory functions.

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ANALYSE DER ELEKTRISCHEN AKTIVITÄT DER NERVEN DER SÜSSWASSERMUSCHEL (*ANODONTA CYGNEA* L.) *IN SITU*

An *Anodonta cygnea* wurde bei extracellulärer Ableitung in situ festgestellt, daß (1) bei Ruhe für die Nerven der Muschel eine Aktivität von relativ geringer Amplitude (unter 40 μV) kennzeichnend ist und nur an einigen interganglionären Nerven sog. »Riesenpotentiale« von 40—90 μV wahrzunehmen sind. (2) Die »spontane« Aktivität schließt ungeordnete Einzellenpotentiale, von ein bis zwei Zellen ausgehende rhythmische Entladungsfolgen, Impulsausbrüche von mehreren sec wie auch summierte Potentiale in sich. Die Mehrzahl der untersuchten peripheren Nerven enthält sowohl afferente wie auch efferente Fasern. Die langen Impulsausbrüche sind zentralnervöser, die aus ein bis zwei Zellen ausgehenden, am hinteren N. pallialis beobachtbaren Entladungsfolgen sind peripherer Herkunft. Die Durchtrennung des hinteren N. pallialis bewirkt eine erhebliche Veränderung im Aktivitätsmuster des distalen Stumpfes. In dem zum vorderen M. adductor ziehenden Nerven war es nicht möglich, eine afferente Impulsation nachzuweisen. (3) Die Überleitung der durch periphere (mechanische) Reize ausgelösten Impulsentladungen auf die interganglionären Nerven geht mit einer Verminderung der Amplitude und der Zeitdauer einher, während beim Austritt in die Peripherie der entgegengesetzte Prozeß vor sich geht. (4) Die Ganglien vermögen sich an die peripheren Reize zu adaptieren, bzw. es können die über die Ganglien führenden Bahnen blockiert werden. Dem letzteren Vorgang dürfte im Koordinationsprozeß des Nervensystems im Sinne einer Überleitung der efferenten Impulse auf die entsprechenden Bahnen eine Rolle zukommen, und er weist auf das Vorhandensein von speziellen Hemmungsmechanismen hin.

ИСЛЕДОВАНИЕ ЭЛЕКТРИЧЕСКОЙ АКТИВНОСТИ НЕРВОВ БЕЗЗУБКИ (*ANODONTA CYGNEA L.*) *IN SITU*

В ходе внеклеточного отведения потенциалов было установлено:

(1) Активность нервов в покое характеризуется относительно низкой амплитудой (ниже 40 μV). От двух межганглионарных нервов можно было отводить иногда т. н. «гигантские потенциалы» (40—90 μV). (2) Спонтанная активность состоит из безпорядочных одноклеточных потенциалов, ряда ритмических вспышок, исходящих от нескольких клеток, залпов с длительностью нескольких секунд и также из суммарных потенциалов. Активность изученных периферических нервов включает в себе в большинстве случаев и афферентые и эфферентные импульсы. Длинные залпы исходят из центральных структур. Ряды ритмических вспышок, полученных из задних паллиальных нервов произходят с перферии. Перерез последних нервов значительно изменяет тип активности отведений от периферического отдела. В нерве, идущем к передней запирающей мышце авторы не смогли обнаружить афферентную импульсацию. (3) Залпы, вызыванные периферическим раздражением, передаются на межганглионарные нервы с затуханием амплитуд и длительности, но, переходя с межганглионарных на периферические нервы происходит противоположный процесс. Время передачи возбуждения занимает около 180—190 мсек. (4) Ганглии могут адаптироваться к периферическому раздражению. С другой стороны пути, переходящие через ганглии, могут блокироваться. Это явление может играть роль в координационных процессах ЦНС путем направления эфферентных импульсов на соответствующие пути, и это же указывает на наличие специальных тормозных механизмов.

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THE MORPHOGENESIS OF CYTOSOMES IN THE NEURONES OF ANODONTA CYGNEA L. (MOLLUSCA, PELECYPODA)

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Synopsis

The origin of cytosomes in the neurones of *Anodonta cygnea* was investigated under normal and experimental conditions. It was found that specially transformed mitochondria represent the first stage of cytosomal morphogenesis. From these starting forms through several transitional stages leads the way to the mature cytosome the substances of which are utilized in the cytoplasm. Under experimental conditions the rate of "emptying" of cytosomes can be increased. Increased emptying is followed by a regeneration phase while transforming mitochondria increase in number and the cytosomal store is re-established. The elements of autophagy can also be found in the cytosomal morphogenesis. The results support the mitochondrial origin of cytosomes. This accounts for the cytosomal localization of mitochondrial enzymes (succinic-dehydrogenase, cytochrome-oxidase) and is in alignment with the view that cytosomes serve as metabolic reserve. In spite of the presence of acid phosphatase activity in the cytosomes, they cannot be considered to be completely identical with the lysosomes.

Introduction

The pigment granules occurring in the invertebrate neurones have been called cytosomes by Nolte and co-workers [15]. The opinions about the significance of these organelles are extremely contradictory, for each of the authors tried to interpret their function on the basis of an arbitrarily selected property. Thus e.g., the cytosomes in the neurones of *Unio pictorum* containing a yellow pigment, were described by Nagy [13, 14] as ageing pigments (lipofuscin). This assumption has been refuted by Lábos and co-workers [12], who proved that the pigment belongs to the lipochrome group and its main component is beta-carotene.

Cytosomes strongly stain with paraldehyde-fuchsin. They were interpreted on this basis as neurosecretory material [1, 2, 7, 8]. This view has also been refuted by several authors [15, 17, 19]. Lane [11], having found an acid-phosphatase activity in cytosomes electron microscopically, included them in the lysosome category. Neither this hypothesis explains, however, all the properties of cytosomes, e.g., their respiratory enzyme activity remains unexplained [19, 23]. According to Nolte and co-workers [15] cytosomes represent some kind of metabolic reserve.

Lacy and Horne [10] assumed the mitochondrial origin of cytosomes and called them lipochondria. Fährmann [7] also called attention to their

possible mitochondrial origin, but, since he published no detail, the question has remained open. The aim of the present investigations was just to clarify the morphogenesis of the cytosomes, which may offer an essential basis for the understanding of their function.

Material and method

The investigations were carried out on adult (10—20 cm long) specimens of *Anodonta cygnea*. The ganglia were fixed in 1.5 per cent OsO_4 buffered with s-collidine [4] and embedded in Durcupan ACM. Sections were cut on LKB Ultrotom III and contrasted with lead citrate according to REYNOLDS [16]. Micrographs were taken with a TESLA BS 413 A electron microscope.

Besides the cerebral, visceral and pedal ganglia of normal animals the cytosomes were investigated in ganglia of reserpine-treated (10 mg/kg) animals. The histochemical and electron microscopial changes observed after this treatment have been published elsewhere [20, 21]. In the present paper only the findings throwing light on the cytosomal morphogenesis are dealt with. Furthermore, the condition of cytosomes was investigated in hypoxic animals having been kept for 2—4 days in water covered with liquid paraffin, as well as in animals having lived some days in reoxygenated water after prolonged anoxia.

Results and discussion

Cytosomes are constant organelles of the neurones in mussels. The commonest properties of cytosomes are shown in Fig. 1. They consist of spots of a material of high electron density as main constituent and a lipoprotein membrane system as well as a fine granulated substance, within a limiting membrane. These components participate in the composition of cytosomes in a very variable proportion, so there is a great variety of forms [22].

Starting from the consideration of LACY and HORNE [10] as well as of FÄHRMANN [7] we searched for the forms that would represent the transition from mitochondria to mature cytosomes. There are two varieties of mitochondria in normal animals [19, 22]. Besides them, however, we found mitochondria differing from the common ones in containing a very fine granulated material of medium electron density in their matrix. This resembles the granulated substance of cytosomes. Usually these mitochondria are larger than the common ones (Fig. 2) and their form is rounded. Closely connected with these mitochondria we frequently see the flattened profiles of the endoplasmic reticulum, which overlying the mitochondria can totally enclose them, forming an outer and an inner sheet (Fig. 2). We consider as the next step of the morphogenesis the forms containing a paracrystalline system at some sites in the granulated matrix (Fig. 3). This can occupy the whole inner structure of some mitochondria (Fig. 4). The paracrystalline system consists of electron-dense lamellae of about 30—50 Å thickness, the interlamellar space being of about 50—80 Å. The accumulation of a homogeneous material of high electron density, obviously of lipid nature, begins on this lamellar system. Sometimes,

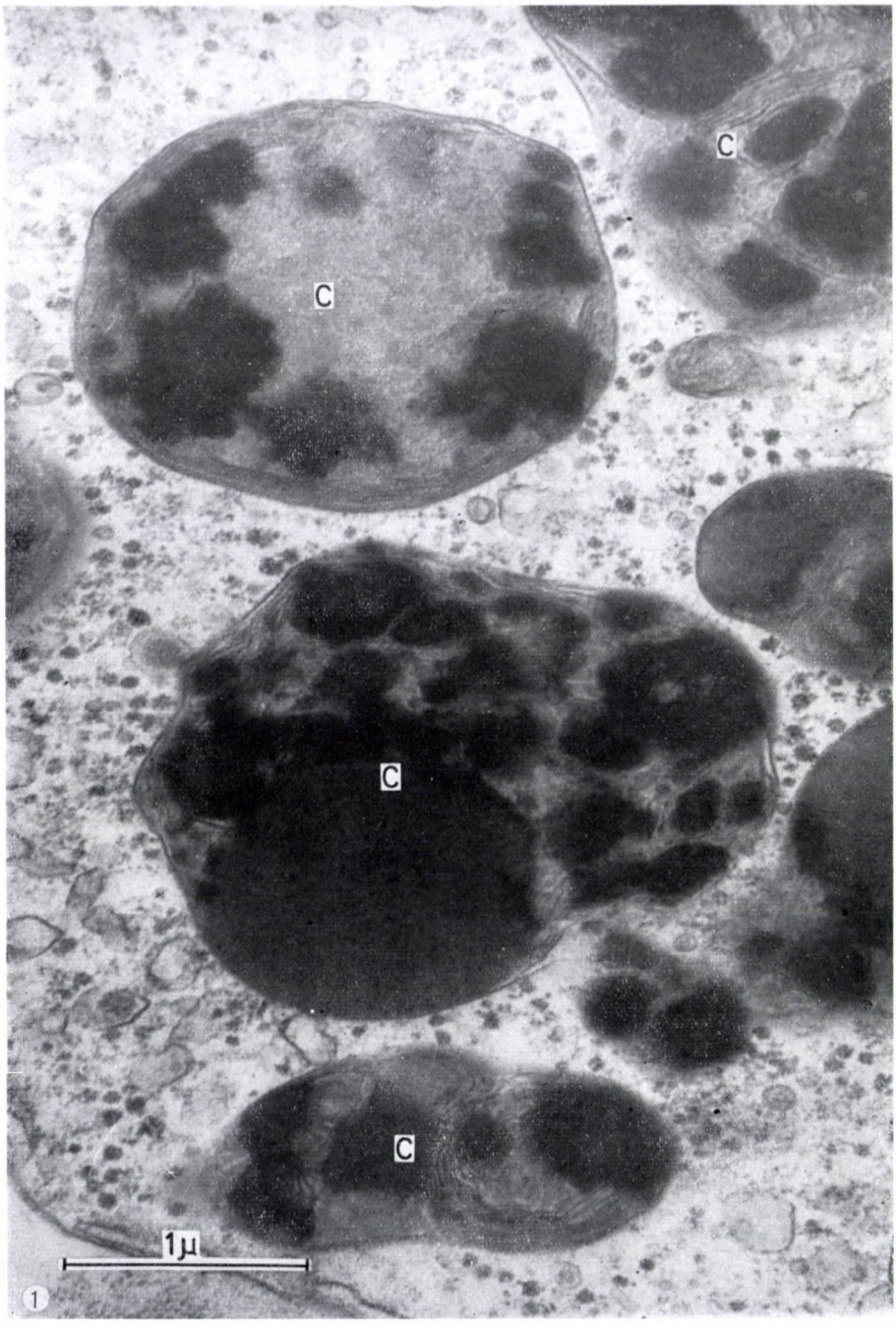

Fig. 1. Normal cytosomes (C) from the cerebral ganglion. ×32,500

when the lipids are less abundant, the paracrystalline pattern rests visible to some extent. The connection with the endoplasmic reticulum remains close.

In the next stage the characteristics of mitochondria completely disappear. This is the reason of calling these forms precytosomes in contradistinction to mitochondria. The original membranes of the mitochondria and those of the endoplasmic reticulum cannot be recognized in this stage. There exists

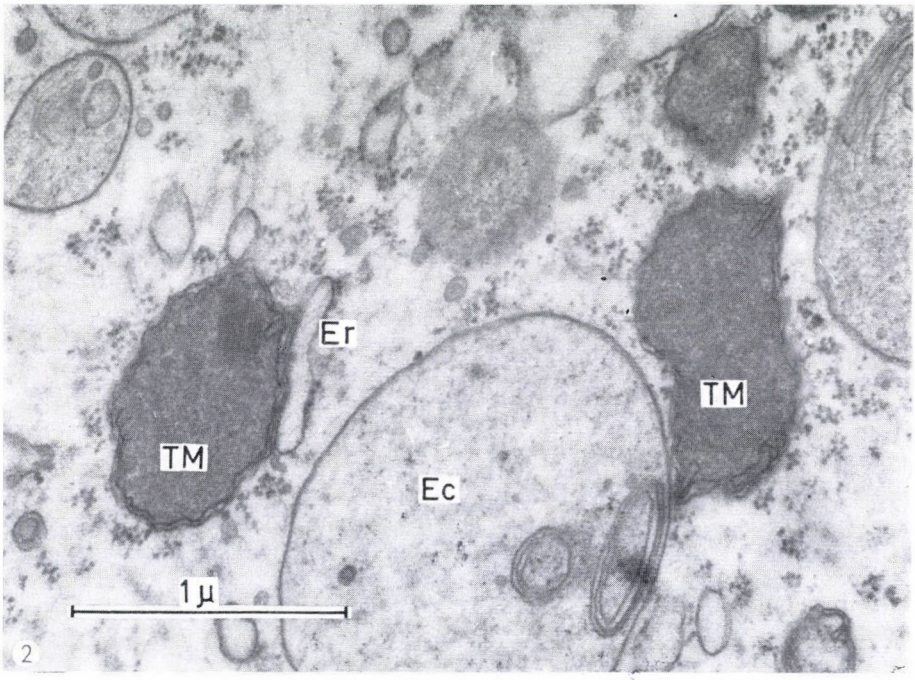

Fig. 2. The first stage of the mitochondrial transformation: accumulation of the granulated material in the matrix (TM). Er = endoplasmic reticulum profile; Ec = "emptying" cytosome. Cerebral ganglion. ×35,000

a wide variety of precytosomes. We consider as the youngest formation the membranes of which are indistinct around the earlier mitochondrial content (Fig. 5a). Later a single unit membrane comes into being, which will correspond to the definitive outer membrane of the cytosome (Fig. 5b). Inside this the membrane formation continues, in consequence of which a manifold concentric membrane system develops (Fig. 5c) while the material of high electron density accumulates in the inside (Fig. 5d). Afterwards the membrane system infolds from the periphery of the precytosome (Fig. 6) and gives rise to different intracytosomal figures. These processes take place obviously at a different rate, resulting in a great variability of precytosomes.

There is no qualitative difference between precytosomes and mature cytosomes (Fig. 1). The first ones are transformed into the latter ones by a further increase of their outer diameter and further accumulation of lipids.

The fate of cytosomes can be explained by the forms, in which the material of high electron density diminishes, the outer membrane breaks out or disappears and the content seems to stream out into the cytoplasm (Fig. 7). Such "emptying" forms of cytosomes can be found always in normal cells,

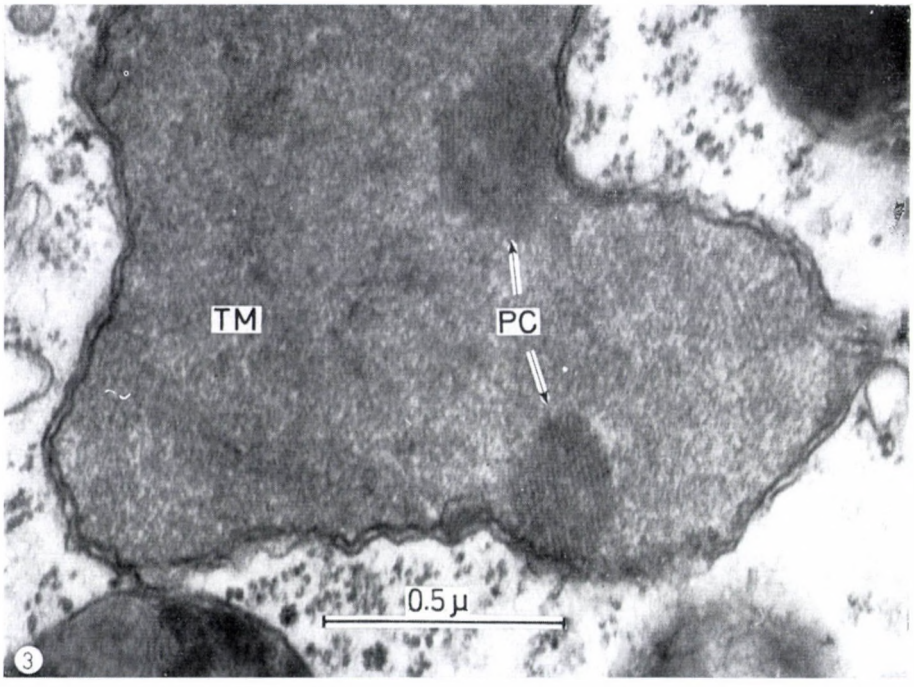

Fig. 3. Paracrystalline structure (PC) occurring sporadically in the transforming mitochondrion (TM). Cerebral ganglion. ×62,400

but the majority of them belongs normally to the mature, quiet form. We have never found cytosomes emptying into the extracellular space.

When the animals were treated with reserpine, in order to deplete the monoamine content of the neurones, a very intensive catecholamine production began in the perikarya of the cerebral ganglion 2—3 days after the reserpine-effect was over [20]. In connection with this the whole cytoplasm became strongly active and striking changes appeared in the cytosomes too [21]. Their majority appeared in the "emptying" form and only few cytosomes remained in normal state. In 6—8 days, when these processes had already been over, there appeared a great number of transforming mitochondria (Fig. 8), which then gradually restituted the cytosomal store.

During a hypoxia of 3—4 days the rate of "emptying" cytosomes had markedly increased (Fig. 9). If this was followed by reoxygenation, the transformation of mitochondria appeared again to a greater intensity and new cytosomes came into being. It is noteworthy that BARANYI and SALÁNKI [3] have already observed the decrease of a paraldehyde-fuchsin-positive substance after 72-hour anoxia in the same experimental conditions. These authors, however, interpreted this phenomenon from the aspect of "neurosecretion."

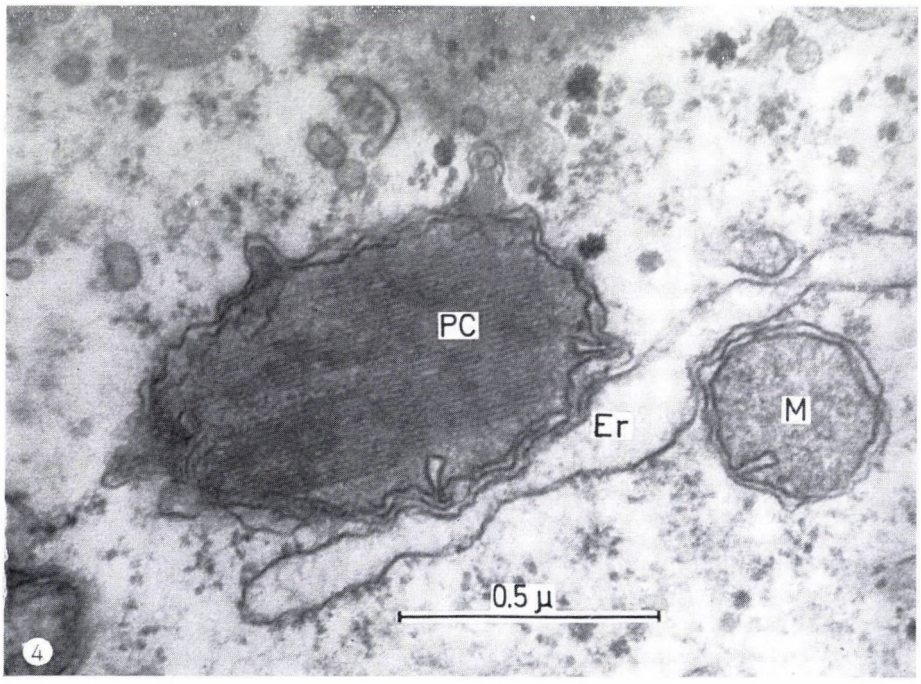

Fig. 4. Paracrystalline structure (PC) occupies a great part of the transforming mitochondrion. Er = endoplasmic reticulum profile; M = mitochondrion showing the initial phase of transformation in its matrix. Cerebral ganglion. ×68,200

In fact, the paraldehyde fuchsin-positive material, decreasing in amount, corresponds to the cytosomal content [19].

We found so in the neurones of normal mussels transformed varieties of mitochondria which represent a morphogenetic series leading from the mitochondria to the cytosomes. It is quite sure that this in itself cannot be considered as evidence for the mitochondrial origin of cytosomes. We are of the opinion, however, that the changes observed under experimental conditions are conclusive. The cytosomal "emptying" caused by quite different effects (regeneration after reserpine-treatment, prolonged anoxia) was always followed during the normalization of essential conditions by an increase of

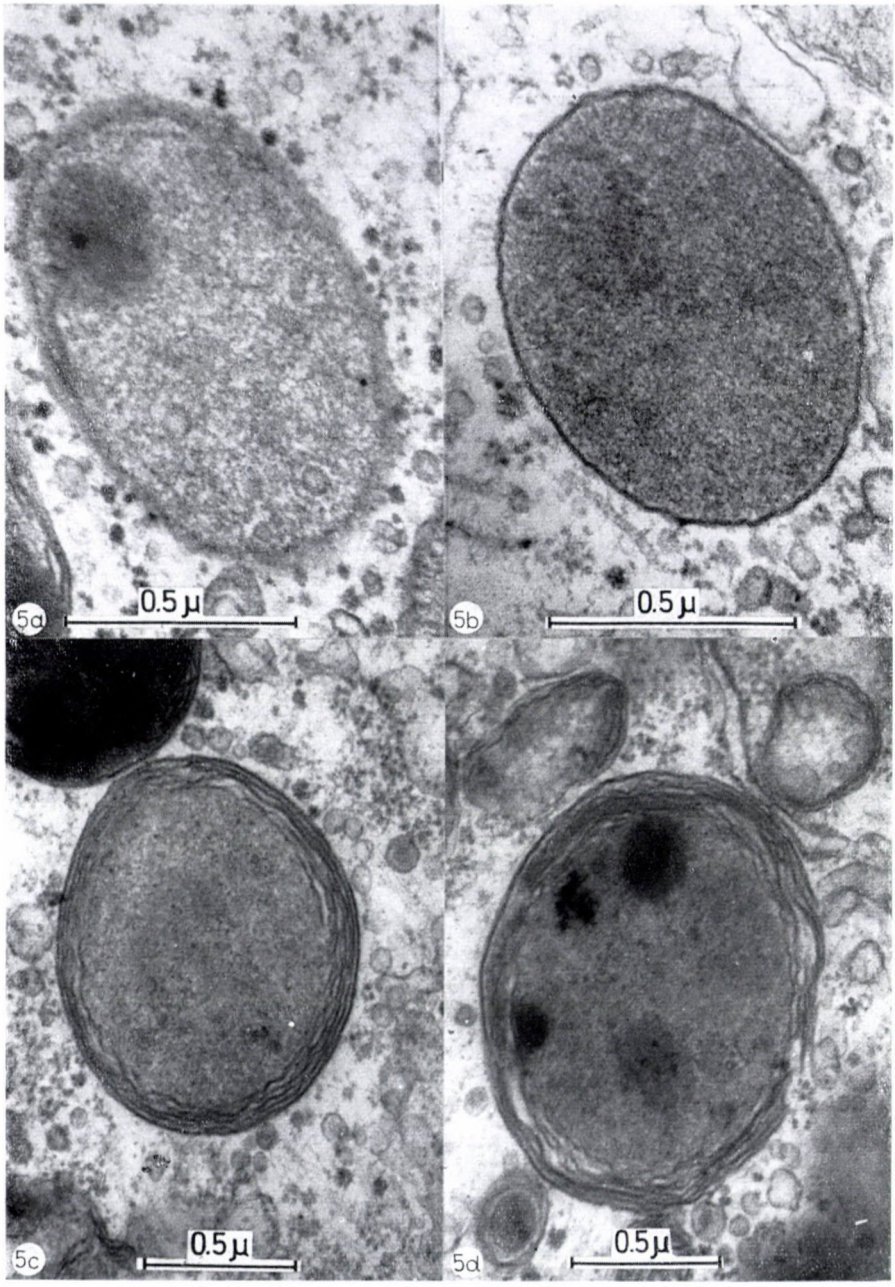

Fig. 5. Various forms of precytosomes from the cerebral ganglion. a = The outer membranes are indistinct. ×61,400; b = A single unit membrane surrounds the precytosome. ×66,000; c = Developed membrane system in the periphery of the precytosome. ×40,000; d = Precytosome showing an accumulation of lipids as well as a concentric membrane system. ×40,000

transforming mitochondria and by the restitution of cytosomal store. Taking also this fact into consideration, we can hold as evident the mitochondrial origin of cytosomes [7, 10] though it should be noted that the membranes of the endoplasmic reticulum also play a great part in this process. It may be stressed, however, that the definitive cytosomal membranes are neoformations.

The mitochondrial origin of cytosomes is supported also by our histochemical investigations: we found significant cytochrome-oxidase and succinic-

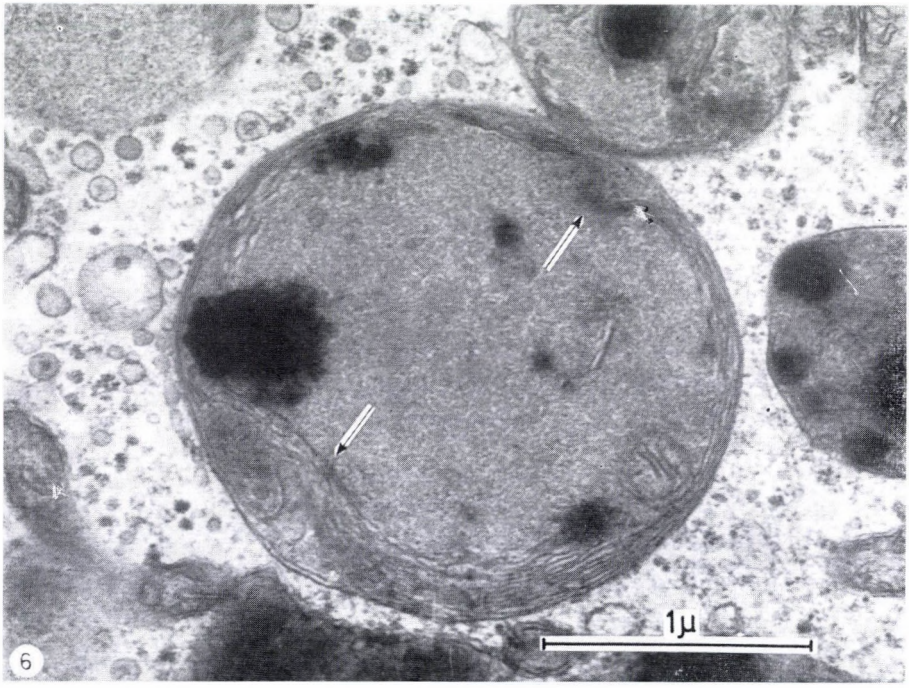

Fig. 6. Picture of a greater precytosome in which the membrane system begins to infold (arrows) from the periphery to the central part. Cerebral ganglion. ×35,000

dehydrogenase activities in cytosomes [19]. The latter enzyme was demonstrated in the lipoprotein membranes of cytosomes by an electron microscopic histochemical method, too [23]. Thus, despite the acid phosphatase activity having been shown by LANE [11], the cytosomes cannot be assumed to be identical with the lysosomes, for lysosomes have never been found to contain respiratory enzymes [9]. The mechanism of cytosomal morphogenesis resembles that of the autophagic vacuoles [5] but in many respects differs from it. E.g., TÖRŐ and VIRÁGH [18] observed in liver cells of starving bats arousing from hibernation a great number of autophagic vacuoles enclosing mitochondria and other organelles; these quickly disappeared on normal feeding.

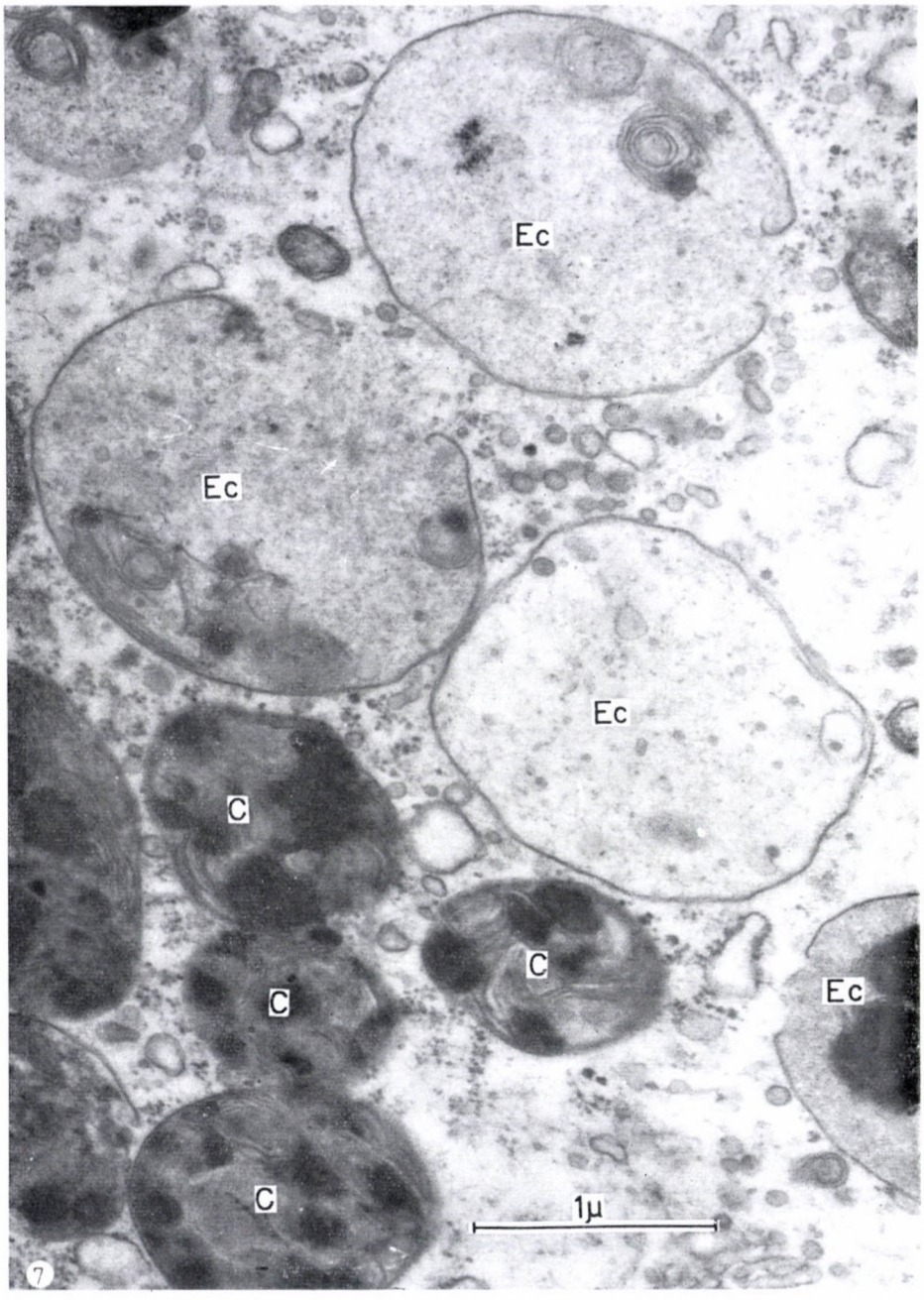

Fig. 7. "Emptying" cytosomes (EC) together with normal ones (C). Cerebral ganglion. ×32,500

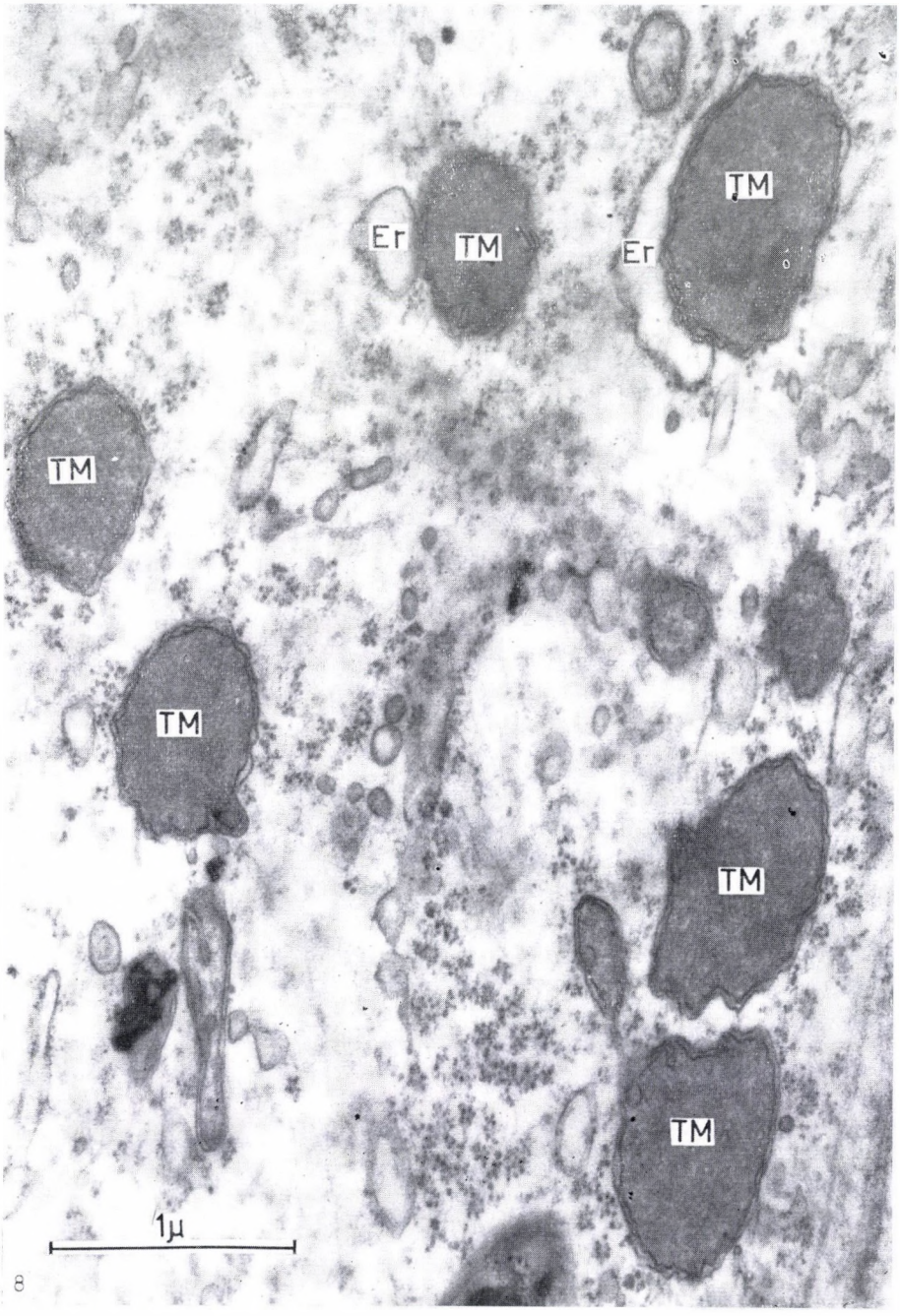

Fig. 8. Eight days subsequent to the reserpine treatment. Note the increased number of transforming mitochondria (TM) and the lack of normal cytosomes. Er = endoplasmic reticulum. Cerebral ganglion. ×32,500

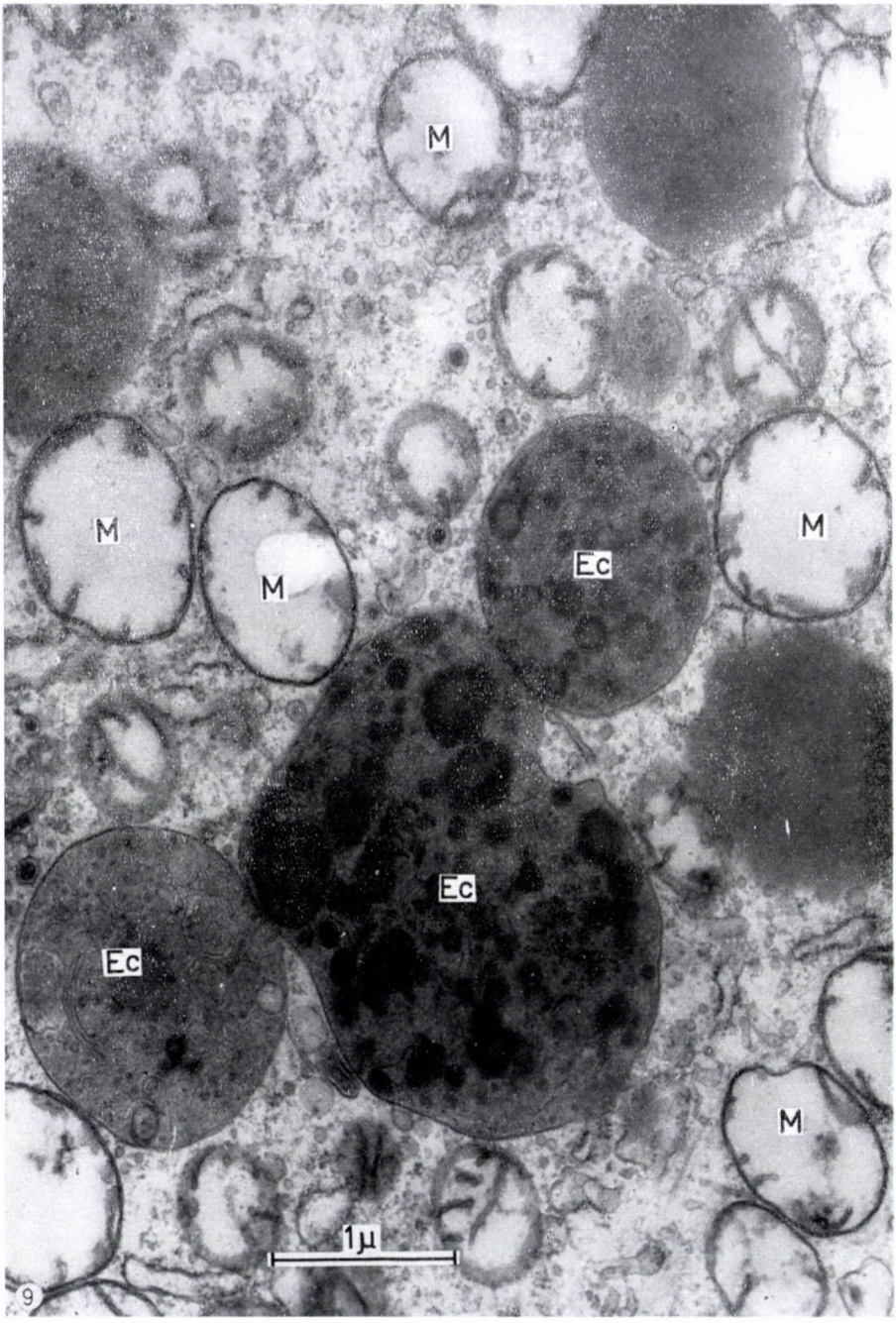

Fig. 9. Changes in the cerebral ganglion, caused by 4-day anoxia. EC = "emptying" cytosomes. The granulating and the disappearance of the material of high electron density belong to the characteristic consequences of anoxia. The swelling of mitochondria (M) is also caused by the anoxia. ×25,000

In the case of mussels a reversed situation exists: the cytosomes come into being in normal feeding, they are constant components of the cytoplasm and are used up only in a hyperactive period of the cell. This clearly shows that in accordance with the theory of NOLTE and co-workers [15], the cytosomes have some kind of general significance and it seems to be justified that cytosomes may serve as metabolic reserve. Although this is not expressed in the name cytosome and this name has already been used for designation of other cell organelles too [6], the maintenance of the name seems to be right, until the function of cytosomes will be better known and an appropriate, unequivocal name will be found for them.

Taking into account that in invertebrates the rather undeveloped circulatory and nutritive systems are hardly able to maintain the proper constant inner milieu, the general occurrence of the cytosomal internal reserve in the neurones of invertebrates becomes understandable, for the neurones obviously are the most exigent cells toward the proper level of metabolism and when the circulatory system is not able to offer this, the neuronal metabolism takes place at the expense of the inner reserve.

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DIE MORPHOGENESE DER CYTOSOMEN IN DEN NEURONEN DER *ANODONTA CYGNEA* L. (MOLLUSCA, PELECYPODA)

Es wurde die Entstehung der Cytosomen in den Neuronen von *Anodonta cygnea* unter normalen und experimentellen Umständen untersucht. Dabei wurde bestätigt, daß spezifisch transformierte Mitochondrien existieren, die einen Ausgangspunkt der Morphogenese von Cytosomen bilden. Von diesen Anfangsformen über mehreren Übergangsfiguren führt der Weg zu den reifen Cytosomen, deren Material im Cytoplasma verbraucht wird. Unter experimentellen Umständen ist ein Zustand herbeizuführen, in dem die »Entleerung« der Cytosomen gesteigert ist. In den nachfolgenden Regenerationsprozessen nimmt die Zahl der sich umformenden Mitochondrien immer zu und die cytosomalen Reserven restituieren sich. Auch die Elementen der Autophagie können in der Morphogenese der Cytosomen aufgefunden werden. Die Untersuchungen beweisen den mitochondrialen Ursprung der Cytosomen. Dadurch erklärt sich die cytosomale Lokalisation der mitochondrialen Enzyme (Cytochrom-Oxydase, Succinodehydrogenase) und das entspricht auch der Stoffwechseldepot-Theorie der Cytosomen. Gleichzeitig zeigen diese Angaben daß trotz einer sauren Phosphatase-Aktivität, die Cytosomen nicht ohne weiteres als Lysosomen betrachtet werden können.

МОРФОГЕНЕЗ ЦИТОСОМ В НЕЙРОНАХ БЕЗЗУБКИ

Был изучен морфогенез цитосом в нейронах беззубки в нормальных и экспериментальных условиях. Установлено, что бывают специфически трансформированные митохондрии, которые служат источником морфогенеза цитосом. От этих исходных форм через несколько переходных фигур ведет дорога к зрелым цитосомам, материалы которых используются в цитоплазме. В экспериментальных условиях вызываются состояния, в которых «опораживание» цитосом повышается и после этого во время регенерации всегда умножаются переходные формы митохондриев и состав цитосом регенерируется. В морфогенезе цитосом наидутся и элементы автофагии. Исследования доказывают митохондриальное происхождение цитосом. Это объясняет локализацию митохондриальных ферментов (цитохромоксидаза, сукцинодегидрогеназа) в цитосомах и соответствует теорией тех, кто считают цитосомы метаболическим резервом. В то же время это показывает, что несмотря на то, что кислая фосфатаза присуствует в цитосомах, нельзя считать их просто лизосомам

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